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AWARD NUMBER: W81XWH-07-1-0351

TITLE: hEcd, A Novel Regulator of Mammary Epithelial Cell Survival

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REPORT DATE: September 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

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<b>REPORT DOCUMENTATION PAGE</b>				Form Approved OMB No. 0704-0188	
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<b>1. REPORT DATE</b> 1 September 2009		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED</b> 29 Aug 2008 – 31 Aug 2009	
<b>4. TITLE AND SUBTITLE</b>  hEcd, A Novel Regulator of Mammary Epithelial Cell Survival				<b>5a. CONTRACT NUMBER</b>	
				<b>5b. GRANT NUMBER</b> W81XWH-07-1-0351	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b>  Vimla Band, Ph.D.  E-Mail: vband@unmc.edu				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  University of Nebraska Medical Center Omaha, NE 68198				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
				<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Using theYeast Two hybrid analysis with human papilloma virus oncogene E6 (the most efficient oncogene to immortalize hMECs in vitro) as a bait and mammary epithelial cell cDNA library, we identified hEcd (human orthologue of Drosophila Ecdysoless) as a novel E6 binding partner. To study the cellular function of Ecd in mammalian cells, we generated Ecd (lox/lox) mouse embryonic fibroblast cells from Ecd floxed mouse embryos (mice generation was not supported by DOD grant) . We observed that Cre-mediated deletion of Ecd in Ecd(lox/lox) mouse embryonic fibroblasts led to a proliferative block due to a delay in G(1)-S cell cycle progression; this defect was reversed by the introduction of human Ecd. Loss of Ecd led to marked down-regulation of E2F target gene expression. Ecd directly bound to Rb at the pocket domain and competed with E2F for binding to hypophosphorylated Rb, demonstrating Ecd plays a role in cell cycle progression via the Rb-E2F pathway. Studies in yeast suggested a potential role of Ecd in transcription; however Ecd lacks a DNA binding domain. Using a GAL4-luciferase reporter assay and a GAL4-DNA binding domain (DBD) fusion with Ecd or its mutants, we present evidence that human Ecd has a transactivation activity in its C-terminal region. We further demonstrate that Ecd interacts with p300, a histone acetyltransferase, and the co-expression of Ecd with p300 enhances the Ecd-mediated transactivation activity. These results demonstrate human Ecd regulates i) cell cycle progression and ii) transactivation probably by interacting with a transcriptional factor. Future studies should discover potential partners of Ecd in this process.					
<b>15. SUBJECT TERMS</b> Breast Cancer, mammary epithelial cells, Ecdysoless, hSGT1					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>  UU	<b>18. NUMBER OF PAGES</b>  38	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b> U	<b>b. ABSTRACT</b> U	<b>c. THIS PAGE</b> U			<b>19b. TELEPHONE NUMBER</b> (include area code)

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## **Title: hEcd, A Novel Regulator of Mammary Epithelial Cell Survival**

### **Introduction:**

The experiments proposed in this idea grant are designed to test the hypothesis that human ecdysoneless (hEcd) is a novel integrator of metabolic pathway responses during cell survival as well as specific biosynthetic pathways. Alteration of hEcd function may therefore help override the metabolic checkpoint in cells and contribute to oncogenic transformation.

We have identified a novel protein using the Yeast Two hybrid analysis with human papilloma virus oncogene E6 (the most efficient oncogene to immortalize hMECs in vitro), designated as hEcd (human orthologue of *Drosophila* Ecdysoneless) as a novel regulator of hMEC survival. So far there are three publications on Ecd protein, two on yeast and one on *drosophila* ortholog [1-3]. First study reported that the Ecd complemented the growth defect in a *Saccharomyces cerevisiae* mutant strain defective in the function of GCR2, which functions as an essential coregulator of GCR1 transcription factor responsible for the induction of glycolytic gene expression under glucose starvation [1,2], thus the human gene was designated as hSGT1 or human suppressor of GCR2 [1,2]. Notably, there is no significant sequence homology between yeast GCR2 and hSGT1 and *S. cerevisiae* in fact lacks a true hSGT1 ortholog whereas hSGT1 orthologs exist throughout eukaryotic species including the yeast *S. pombe* [1,2]. However, hEcd complemented the *S. cerevisiae* GCR2 mutation and restored glycolytic gene expression [2]. Microarray analysis in *S. pombe* with a mutant of Ecd revealed that Ecd regulates the expression of genes involved in carbohydrate, amino acid and lipid metabolism [2]. A recent paper in *Drosophila* shows a cell-autonomous cell survival of Ecd in addition to its critical function in ecdysone synthesis [3] Ecd mutant in *drosophila* was known for many years for lack of ecdysone synthesis, apparently due to defect in the transport of cholesterol between different subcellular compartments [4,5] but the gene was not known until recently [3]. Notably, a prominent phenotype induced by mutant Ecd in *Drosophila* was defect in molting, a known phenomenon that needs cholesterol [6]. Given the existence of an unrelated family of well-characterized SGT1 proteins [7] and the identification of the well-known ecdysoneless gene product as hSGT1 ortholog, we will use the Ecd designation and refer to human Ecd as hEcd. We have recently shown that hEcd binds to p53, stabilizes the protein and enhances p53-mediated functions [8]. To assess the functional role(s) of hEcd, we designed two RNAi sequences that led to substantial knockdown of hEcd protein expression in normal human epithelial cells. Contrary to our initial expectation from its p53 regulatory role suggested by overexpression studies and cell proliferation upon transient knockdown, stable hEcd knockdown did not lead to increased proliferation of hMECs. Instead knockdown of Ecd led to inhibition of cell proliferation. Notably, the hEcd RNAi phenotype was independent of p53 as it was observed in p53-positive as well as p53-negative cells. These results indicated that hEcd plays a p53-independent role in cell proliferation. The phenotypes of ecdysoneless mutant *Drosophila* [3] and Ecd-null *S. pombe* [1,2] support this conclusion. Studies in yeast, *drosophila* and our initial observation suggest a novel cell

survival function of hEcd probably through its evolutionarily-conserved roles in regulating the availability of glucose and other nutrients.

A synthesis of the findings discussed above leads us to hypothesize that is proposed above. To test the hypothesis, we proposed two specific aims

### **AIM I. DETERMINE THE STRUCTURAL BASIS OF hEcd-MEDIATED METABOLIC CELL SURVIVAL FUNCTION**

I-A. Rescue the lethality of RNAi knockdown of hMECs by expressing RNAi-resistant Ecd mutants.

I-B. Define the domains of hEcd involved in cell survival pathway.

### **AIM II. DISSECT THE BIOCHEMICAL BASIS OF THE ROLE OF Ecd IN CELL SURVIVAL**

II-A. Assess if hEcd participates in metabolic cell survival through AMPK.

II-B. Identify cellular targets of potential transcriptional regulatory function of Ecd using microarray analyses.

II-C. Identify binding partners of hEcd using the tandem affinity purification (TAP) and yeast-two hybrid system.

### **Body:**

Human Ecd is a novel protein of unknown function. Our hypothesis based on yeast Ecd speculated its role in metabolic cell survival pathway however we have consistently not observed any effect on metabolic pathways upon Ecd removal or overexpression. While we continue to change our experimental conditions to examine if Ecd has a role in metabolic pathway, we have made significant progress in understanding the physiological functions of Ecd. We consistently observed that lack of Ecd induced cell cycle arrest. Based on this observation and the fact that knockdown of Ecd in mammary epithelial cells significantly reduced expression of E2F target genes (our microarray analysis reported in last year's annual report), we examined two functions of Ecd i) its role in cell cycle and ii) its role in transcription. Both of these findings have led to significant insights in functions of Ecd, as described below (details are in two attached publications).

### **Role of mammalian Ecdysoneless in cell cycle regulation.**

The Ecdysoneless (Ecd) protein is required for cell-autonomous roles in development and oogenesis in *Drosophila*, but the function of its evolutionarily conserved mammalian orthologs is not clear. To study the cellular function of Ecd in mammalian cells, we generated Ecd conditional knockout mice that turn out to be embryonically lethal, suggesting an essential role of Ecd in cell survival (Generation of Ecd knockout mice was not funded by DOD grant but was supported by institutional start-up funds to the P.I.). Next, we generated Ecd(lox/lox) mouse embryonic fibroblast cells from Ecd floxed mouse embryos. We observed that Cre-mediated deletion of Ecd in Ecd(lox/lox) mouse embryonic fibroblasts led to a proliferative block due to a delay in G(1)-S cell cycle progression; this defect was reversed by the introduction of human Ecd. Loss of Ecd led

to marked down-regulation of E2F target gene expression. Furthermore, Ecd directly bound to Rb at the pocket domain and competed with E2F for binding to hypophosphorylated Rb. Our results demonstrate that mammalian Ecd plays a role in cell cycle progression via the Rb-E2F pathway. For details, please see attached manuscript (Kim JH, Gurumurthy CB, Naramura M, Zhang Y, Dudley AT, Doglio L, Band H, Band V. J Biol Chem. 2009 Sep 25;284(39):26402-10.

### **Biochemical characterization of human Ecdysonesless reveals a role in transcriptional regulation.**

Ecdysonesless (Ecd) is an evolutionarily conserved protein whose function is essential for embryonic development in *Drosophila* and cell growth in yeast. However, its function has remained unknown until recently. Studies in yeast suggested a potential role of Ecd in transcription; however Ecd lacks a DNA binding domain. Using a GAL4-luciferase reporter assay and a GAL4-DNA binding domain (DBD) fusion with Ecd or its mutants, we present evidence that human Ecd has a transactivation activity in its C-terminal region. Importantly, further analyses using point mutants showed that a single amino acid change at either Asp-484 or Leu-489 essentially completely abolishes the transactivation activity of Ecd. We further demonstrate that Ecd interacts with p300, a histone acetyltransferase, and the co-expression of Ecd with p300 enhances the Ecd-mediated transactivation activity. Ecd localizes to both nucleus and cytoplasm and shuttles between the nucleus and cytoplasm; however it exhibits strong nuclear export. Based on previous yeast studies and evidence provided here, we suggest that Ecd functions as a transcriptional regulator. Our results indicate an important function of human Ecd and provides a basis to explore the transcriptional partners of Ecd. For details, please see attached manuscript (Kim JH, Gurumurthy CB, Band H, Band V. Biol Chem. 2009 Nov 17)

### **Goals for the current year:**

- We will continue to examine the role of Ecd in cell cycle by shRNA knockdown or overexpression in mammary epithelial cells.
- We will examine the role of Ecd in oncogenesis by removing or overexpressing Ecd in immortal and tumor mammary epithelial cells
- We will make an attempt to identify transcriptional partner for Ecd as Ecd itself is not a transcriptional factor (it lacks DNA binding domain).
- We will initiate experiments to identify Ecd-interacting proteins.

### **Key Research Accomplishments:**

1. We have demonstrated a role of mammalian Ecdysoneless in cell cycle regulation.
2. Biochemical characterization of human Ecdysoneless reveals a role in transcriptional regulation.

#### **Reportable Outcomes:**

Kim JH, Gurumurthy CB, Naramura M, Zhang Y, Dudley AT, Doglio L, Band H, Band V. J Biol Chem. Role of mammalian Ecdysoneless in cell cycle regulation. 2009 Sep 25;284(39):26402-10.

Kim JH, Gurumurthy CB, Band H, Band V. Biochemical characterization of human Ecdysoneless reveals a role in transcriptional regulation. 2009 Nov 17. Biol. Chem

#### **Reagents:**

-Generated Ecd mutants.

**Funding applied for based on this work:** An RO1 application is submitted to the NCI.

**Manuscript included:** Yes. Two manuscripts are attached.

**Conclusions:** In conclusions, our results demonstrate that Ecd plays a role in cell cycle progression via the Rb-E2F pathway and Ecd functions as a transcriptional regulator. Future studies should determine if cell cycle regulatory function of Ecd is due to its being transcriptional regulator or these are two independent functions of Ecd.

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# THE ROLE OF MAMMALIAN ECDYSONELESS IN CELL CYCLE REGULATION

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The work presented here was initiated while the investigators (JHK, CBG, MN, YZ, HB and VB) were at: Department of Medicine, Evanston Northwestern Healthcare Research Institute, Northwestern University, Evanston, IL

Running head: a novel Rb-E2F pathway regulator / Ecd

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**Ecdysonless protein is required for cell-autonomous roles in development and oogenesis in *Drosophila* but the function of its evolutionarily conserved mammalian orthologues is not clear. In order to study the cellular function of Ecd in mammalian cells, we generated *Ecd*<sup>lox/lox</sup> mouse embryonic fibroblast cells from *Ecd* floxed mouse embryo. Cre-mediated deletion of Ecd in *Ecd*<sup>lox/lox</sup> MEFs led to a proliferative block due to a delay in G<sub>1</sub>-S cell cycle progression; this defect was reversed by the introduction of human Ecd. Loss of Ecd led to marked downregulation of E2F target gene expression. Furthermore, Ecd directly binds to Rb at the pocket domain, and competes with E2F for binding to hypophosphorylated Rb. Our results demonstrate that mammalian Ecd plays a role in cell cycle progression via Rb-E2F pathway.**

Precisely regulated cell proliferation is essential for embryonic development as well as for homeostasis in adult organs and tissues, whereas uncontrolled cell proliferation is a hallmark of cancer. Thus, understanding how the cell cycle machinery is controlled is an important area of research.

Studies with viral oncogenes that induce dominant cellular transformation have led to the identification and elucidation of a number of

biochemical pathways that are perturbed in human cancer (1). One group of tumor viruses directly implicated in the pathogenesis of human cancer is the "high-risk" subgroup of human papillomaviruses (HPVs). *In vitro* studies have defined two HPV oncogenes, E6 and E7, that are nearly always expressed in HPV-associated carcinomas and cell lines derived from them (2). The ability of HPV E6 and/or E7 oncogenes to induce the immortalization of human epithelial cells *in vitro* has provided an invaluable tool to elucidate the mechanisms by which these oncogenes function (1-3).

Recently, we identified the human orthologue of *Drosophila* Ecdysonless (hEcd) as an E6-binding partner and our initial studies indicated that hEcd interacts with and stabilizes p53. Furthermore, its overexpression in mammalian cells enhanced the p53 target gene transcription whereas its transient knockdown had the opposite effect (4). However, the physiological role of hEcd remains unknown. Notably, unlike stable knockdown of p53 (which extended the life span of normally senescing cells), stable hEcd knockdown led to a proliferative block, suggesting a p53-independent role for hEcd in cell survival/proliferation.

The *Ecd* gene was defined nearly thirty years ago based on *Drosophila ecdysonless (ecd)* mutations ; temperature-sensitive *ecd* mutant

embryos arrest at 2<sup>nd</sup> larval instar stage at the restrictive temperature, apparently due to reduced levels of ecdysone (5). The protein product of this locus was molecularly identified only recently and *ecd* mutants were found to exhibit a general defect in cell survival in addition to the reproductive and developmental defects; the nature of the survival defect or its biochemical basis remains unknown (6). Two studies in yeast suggest that Ecd (called hSGT1 in these studies) might be involved in the transcription of glycolytic genes. Sato *et al.* identified human Ecd (hEcd) using a cross-species complementation where hEcd was shown to rescue the growth defect of the *gcr2* mutation in which transcription of glycolytic genes is dysregulated (7). The authors concluded that human Ecd/hSGT1 could have a role in gene transcription; however, the human Ecd/hSGT1 has no sequence similarity with GCR2 gene whose deficiency it complemented and in fact *S. cerevisiae* does not have a hEcd orthologue. In a follow up study, the same investigators identified an Ecd orthologue in *S. pombe* and found it to be essential for yeast survival and growth; the mechanisms of its function, however, remain unknown (8).

Given the limited understanding of the evolutionarily-conserved Ecd protein family, we generated conditional Ecd knockout mice and examined the consequences of Ecd deletion. We observed that homozygous Ecd deletion in mice leads to early embryonic lethality (manuscript in preparation). In order to examine the role of Ecd at cellular level, we generated *Ecd*-null mouse embryonic fibroblasts (MEFs) and then deleted Ecd by adenovirus-Cre infection of *Ecd*<sup>lox/lox</sup> MEFs. *Ecd*-deleted cells showed a delay in G<sub>1</sub>-S cell cycle progression with a delay in Rb phosphorylation and reduced expression of E2F target genes.

The retinoblastoma (Rb) protein family, consisting of three related proteins Rb/p105, p107 and Rb2/p130, has emerged as a key controller of cell cycle progression and these proteins play critical roles in development and differentiation of various tissues (9-16). Precisely how the Rb-family proteins regulate cell proliferation is still incompletely understood. A large body of evidence has established a basic paradigm of how these proteins contribute to the control of cell cycle progression. Unphosphorylated Rb proteins

interact with E2F transcription factors and prevent the transcription of genes regulated by E2F proteins (17,18). During cell cycle progression, cyclin-CDK complexes mediate hyperphosphorylation of Rb, an event that leads to loss of its interaction with E2F; this allows E2F target gene transcription and cell cycle progression.

In this manuscript, we provide evidence that Ecd directly interacts with Rb at the pocket domain, competes with E2F for association with hypophosphorylated Rb, and regulates E2F target gene expression and cell cycle progression. Thus, this study demonstrates that mammalian Ecd plays a role in cell cycle progression via Rb-E2F pathway.

## EXPERIMENTAL PROCEDURES

**Establishment of MEFs-** E13.5 embryos were dissected from *Ecd*<sup>+/lox</sup> intercrossed females and MEFs were isolated and immortalized following the 3T3 protocol (19). MEFs were maintained in DMEM supplemented with 10% fetal calf serum. *Ecd*<sup>lox/lox</sup> MEFs expressing human Ecd and HPV 16 E7 were generated by infecting retrovirus encoding full-length human Ecd and HPV 16 E7 genes, respectively. E7 expression was confirmed by RT-PCR using a primer set (5'-GATCTCTACTGTTATGAGCA-3' and 5'-TAACAGGTCTTCCAAAGTAC-3')

**Plasmids-** N-terminal Flag-tagged full-length Ecd (amino acid 1-644) plasmid has been described previously (4). N-terminal Flag-tagged Ecd fragments were generated by PCR amplification and subcloned into EcoRV and NotI sites of pcDNA3.1 (Invitrogen, Carlsbad, CA). For expression of GST-fused Ecd, amino acid 1-644, 150-644 and 439-644 regions of Ecd cDNA were PCR amplified and subcloned into SalI and NotI sites of pGEX-6p-1 (GE Healthcare, Piscataway, NJ). pGEX6p-1-Rb (amino acid 379-928, 379-792, 768-928 and 792-928) and pGEX6p-1-p130 (amino acid 417-1139) were generated by PCR cloning. For expression of C-terminally tagged 6X histidine Ecd, full-length Ecd coding sequence was cloned in to SalI and NotI sites of pFastBac1 Vector (Invitrogen). Protein was expressed in Sf21 cells and purified using Histidine affinity purification.

**Growth curve, colony formation assay and cell cycle analysis** -Adenoviruses encoding EGFP-Cre or EGFP were purchased from the University of

Iowa (Gene transfer Vector core). Adenovirus-infected cells were plated ( $1 \times 10^4$  cells per well of 6 well plates) and counted at the indicated time points. For colony formation assay, the cells were trypsinized 2 days after infection and plated at 5000 cells per 100-mm dish in triplicate or at 1000 cells per well of a 6-well plate. After 10 days, the colonies were stained with crystal violet (0.5% crystal violet in 25% methanol). The stain retained in the colonies was solubilized in 10% acetic acid and absorbance was measured at 590 nm. For BrdU staining, adenovirus-infected cells on coverslips were starved in DMEM containing 0.2% fetal calf serum for 72 h and stimulated with complete DMEM containing 10% fetal calf serum. Cells were labeled with 10  $\mu$ M BrdU for 1 hr and analyzed using mouse anti-BrdU (555627, BD Pharmingen, San Diego, CA). Cells on coverslips were fixed in 1 % paraformaldehyde for 20 min, and then subjected to permeabilization (in PBS containing 0.5 % Triton X-100, for 15 min at RT) and blocking (in PBS containing 1 % BSA, for 30 min at RT). After blocking, cells were incubated for 1 hour with mouse anti-BrdU (1:1000 dilution in PBS containing 0.1 % Tween-20, 0.15 M NaCl, 4.2 mM  $\text{MgCl}_2$ , and 10 Kunits/ml of DNase I) and then with anti-mouse IgG Alexa Fluor 564 (1:1000 dilution) (Molecular Probes) in PBS containing 0.1 % Tween-20. After washing in PBS containing 0.1 % Tween-20, cells on coverslips were mounted using Vectashield solution containing DAPI (Vector Laboratories) and visualized under fluorescence microscope. TUNEL assay was performed using an *in situ* cell death detection kit, POD (Roche)

**Immunoblotting and immunoprecipitation**-Cell extracts were prepared in lysis buffer (80 mM Tris-HCl [pH 6.8], 2% SDS and 5% glycerol) and boiled at 95 °C for 5 minutes and protein concentration was measured using the BCA protein assay reagent (Pierce). Immunoblotting was performed with primary antibodies against Ecd (4A8; generated through the Monoclonal Antibody Facility at the Lurie Cancer Center, Northwestern University, Chicago, IL), Rb (554136, BD Pharmingen), p107 (sc-318, Santa Cruz Biotechnology, Santa Cruz, CA), p130 (sc-317), cyclin A (sc-596), cyclin B1 (sc-752), Cyclin E (sc-481), Cyclin D1 (sc-20044), CDK2 (sc-6248), CDK4 (sc-260), CDK6 (sc-53638) and  $\alpha$ -tubulin (T6199, Sigma, St. Louis, MO). For

immunoprecipitation, cell extracts were prepared in lysis buffer (20 mM Tris-HCl [pH7.5], 200 mM NaCl, 0.5 % NP-40 and a protease inhibitor cocktail from Roche) and immunoprecipitated with 2  $\mu$ g of antibodies against Rb (sc-50 and sc-7905), E2F1 (sc-193), E2F2 (sc-633), and E2F3 (sc-878) for 2 h to overnight at 4°C. The immunocomplexes were pulled down with protein A/G agarose (sc-2003) for an additional 2 h.

**In vitro kinase assay**-*In vitro* kinase assay was performed using purified GST-Rb (amino acid 379-928) as a substrate. Adenovirus-infected MEFs were starved for 3 days and stimulated with serum. Cells were harvested in lysis buffer (20 mM Tris-HCl [pH7.5], 150 mM NaCl, 0.5% NP-40, 0.1 mM  $\text{Na}_4\text{VO}_3$ , 1 mM NaF and protease inhibitor cocktails) and CDK complex was recovered by immunoprecipitation with 2  $\mu$ g of anti-CDK4 (sc-56277), or CDK2 (sc-6248) antibodies. To examine the effects of added Ecd, 2  $\mu$ g of purified Ecd protein was added into 500  $\mu$ g of *Ecd*-null MEF extracts before immunoprecipitation for 2 h at 4°C. CDK2 complex was captured with protein A/G agarose for 1 h, washed with lysis buffer followed by one wash with the kinase buffer (50 mM Tris-HCl [pH 7.5], 7.5 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 0.1 mM  $\text{Na}_4\text{VO}_3$ , 1 mM NaF). CDK2 complex and GST-Rb (500 ng) were incubated in the kinase buffer including 10 mM  $\beta$ -glycerophosphate, 33  $\mu$ M ATP and 10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP [10 mCi/ml; 6,000 Ci/mmol] at room temperature for 20 min. The products were run on SDS-PAGE, transferred to PVDF membranes and autoradiographed.

**In vitro binding assay**-Various GST fusion proteins were purified using glutathione-Sepharose-4B beads (GE Healthcare). GST part was removed by cleavage with PreScission protease (GE Healthcare). GST-pull down assays were performed in the lysis buffer (20 mM Tris-HCl [pH7.5], 150 mM NaCl, 0.5 % NP-40 and protease inhibitor cocktails) for 2 h at 4°C. Flag-tagged Ecd or E2F1 proteins for GST-pull down assays were transiently expressed in 293T or U2OS cells. His-pull down assay was performed in lysis buffer containing 5 mM imidazole with Ni-beads (Invitrogen).

**Real-time PCR**-RNA was isolated from the MEFs infected with control or adeno-Cre viruses using TRIzol reagent (Invitrogen, Carlsbad, CA), and 2

μg of total RNA were used for reverse transcriptase reaction using SuperScript™ II Reverse Transcriptase (Invitrogen). PCR amplification was performed with specific primer sets (see Supplementary Table 1).

**Chromatin immunoprecipitation (ChIP) assay-** ChIP assays were performed using ChIP-IT™ Express assay kit (Active Motif, Carlsbad, CA). Two microgram of antibodies was used for ChIP and PCR amplifications were performed using 35-40 cycles. The following antibodies were used; control IgG (sc-2027) Rb (sc-50), p130 (sc-317). The PCR primers for B-myb, cdc2 were previously described (20). PCR was performed with different PCR cycle number to confirm PCR results from the linear range.

## RESULTS

***Ecd deletion leads to a proliferation defect in MEFs.*** To assess the impact of Ecd deletion at the whole organism level, we generated an Ecd floxed allele by introducing loxP sites flanking exons 4 through 7 to make Ecd knockout mice. When heterozygous *Ecd*<sup>+/-</sup> mice were intercrossed, no *Ecd*-null pups were observed among over 200 live born pups screened, suggesting that homozygous Ecd knockout mice are lethal (manuscript in preparation).

In order to study the role of Ecd at the cellular level, we established spontaneously immortal mouse embryonic fibroblasts (MEF) lines from *Ecd*<sup>+/+</sup> and *Ecd*<sup>lox/lox</sup> mice and generated *Ecd*-null MEFs from the latter by adeno-Cre virus infection. In several experiments, we noted that adenoviral Cre expression in *Ecd*<sup>lox/lox</sup> but not in *Ecd*<sup>+/+</sup> MEFs led to a proliferative block and any residual proliferating cells were found to express Ecd protein reflecting cells that were not adenovirally infected (data not shown). To assure that the proliferative block was indeed a result of Ecd deletion, we engineered *Ecd*<sup>lox/lox/hEcd</sup> MEFs line by retrovirally introducing the human *Ecd* (hEcd) coding sequence into *Ecd*<sup>lox/lox</sup> MEFs. As compared to wild type MEFs, Cre-mediated deletion of Ecd in *Ecd*<sup>lox/lox</sup> MEFs induced a clear proliferative block (Fig. 1A). In contrast, proliferation of *Ecd*<sup>lox/lox/hEcd</sup> MEF was minimally affected (Fig. 1A) even though the endogenous (murine) Ecd was fully deleted (Fig. 1C; upper band). Colony formation assays further confirmed

the proliferative defect upon Ecd deletion and rescue with hEcd (Fig. 1B). These results demonstrate an essential role for Ecd in cell proliferation.

***Ecd deletion leads to a delay in G<sub>1</sub>-S progression.*** We reasoned that the defect in proliferation in *Ecd*-null MEFs could be either due to increased cell death or impairment of cell cycle progression. We excluded the first possibility since we did not observe the characteristic morphological features of apoptosis (Fig. 2A) or any evidence of apoptosis by TUNEL staining (Fig. 2B) in *Ecd*-null MEFs. Notably, *Ecd*-null MEFs exhibited an enlarged and flattened morphology that is typical of G<sub>1</sub>-arrested cells (Figure 2A). We therefore compared the G<sub>1</sub>-S cell cycle progression of *Ecd*-null vs. control MEFs by bromodeoxyuridine (BrdU) staining. *Ecd*<sup>lox/lox</sup> MEFs were infected with control or Cre adenoviruses, synchronized by serum-deprivation and released into cell cycle by adding serum-containing medium. Compared to serum-stimulated *Ecd*<sup>lox/lox</sup> MEFs infected with control virus, adeno-Cre infected cells exhibited a delay in G<sub>1</sub>-S progression (Fig. 2C). These results indicate that the cell proliferation defect found in *Ecd*-null MEFs is due to a delay in G<sub>1</sub>-S progression.

***Ecd deletion leads to impairment of cell cycle-associated Rb phosphorylation.*** Given our observations that lack of Ecd imposed a block in G<sub>1</sub>-S transition, we examined the status of key proteins known to control this transition. It is well established that a critical event during G<sub>1</sub>-S transition is the phosphorylation of Rb by CDK4/6 and CDK2, which leads to the release of E2F family members from the Rb/E2F complex and allows E2Fs to facilitate the expression of E2F-responsive genes (21,22). Therefore, we compared the phosphorylation status of Rb-family proteins in control and *Ecd*-null MEFs. Analysis of unsynchronized cells revealed relatively a moderate increase in the levels of hypophosphorylated as compared to hyperphosphorylated Rb in *Ecd*-deleted vs. control MEFs (Fig. 3A). When synchronized MEFs were serum-stimulated, an expected time-dependent increase in the proportion of hyperphosphorylated Rb family proteins was seen in control cells whereas the emergence of hyperphosphorylated forms was delayed in *Ecd*-null MEFs (Fig. 3B). In

addition, the level of p107 was reduced in *Ecd*-null cells; as discussed later, this is likely because p107 is a transcriptional target of E2F (18).

Next, we compared the control and *Ecd*-null MEFs for the levels of CDK2, CDK4 and CDK6 kinases known to mediate Rb phosphorylation during G<sub>1</sub>-S progression. While CDK4 and CDK6 levels were not altered by *Ecd* deletion, CDK2 expression was reduced in *Ecd*-null MEFs (Fig. 3C). *In vitro* kinase assay showed that while CDK4 kinase activity was comparable between control and *Ecd*-null MEFs (Fig. 3D), the total levels of CDK2 kinase activity in *Ecd*-deleted MEFs were substantially reduced in *Ecd*-null MEFs compared to that of control MEFs (Fig. 3E). Addition of purified Ecd to *Ecd*-null MEF extracts prior to CDK2 immunoprecipitation did not affect the level of CDK2 kinase activity (Fig. 3E). This result suggests that lack of Ecd is unlikely to influence the CDK2 activity directly or by altering its association with cyclins or CDK inhibitor proteins. Furthermore, the expression levels of CDK inhibitors, p16, p21 and p27 were comparable between control and *Ecd*-null MEFs (Fig. 3C). Taken together, these results support the notion that depletion of Ecd protein reduces the expression and activity of CDK2. As CDK2 is a known E2F target and CDK2 promoter has an E2F binding element (23-25), this finding led us to further analyze whether other E2F target genes are down-regulated in *Ecd*-null cells.

***Expression of E2F target genes is reduced in Ecd-null MEFs due to inefficient Rb proteins dissociation from cell cycle gene promoters.*** In view of the delay in cell cycle progression and Rb hyperphosphorylation upon *Ecd* deletion, together with decreased CDK2 levels as well as kinase activity in *Ecd*-deleted cells, we examined the expression of several cell cycle regulatory proteins including those known to be induced through E2F-dependent transcription. The levels of cyclin E, A, and B1 were substantially lower in *Ecd*-deleted MEFs (Fig. 4A). Reconstitution of Ecd expression in *Ecd*<sup>lox/lox</sup> MEFs infected with adeno-Cre virus restored cyclin A expression (Fig. 4B). Real-time-PCR analysis showed a parallel reduction in the mRNA levels of these cyclins (Fig. 4C). The level of cyclin D1, a regulatory component for CDK4, however was comparable in control vs. *Ecd*-null MEFs (Fig. 4A). Since cyclins E1, A and B1 are known E2F target genes, these results (together

with reduction in CDK2 levels and Rb phosphorylation) suggested a role for Ecd in regulating the Rb-E2F pathway.

To further address this notion, we examined the expression of other known E2F target genes, PCNA, B-myb, FoxM1, p107, Birc5, and Cdc2. Notably, these E2F target genes were also downregulated in *Ecd*-null MEFs, while cyclin D1, and GAPDH were not affected, suggesting the specific effect of *Ecd* deletion on E2F-specific targets (Figure 4C). These results clearly indicate that Ecd regulates E2F target gene expression at the transcriptional level.

Given that *Ecd* deletion led to a down-regulation of E2F target genes and reduced the cell cycle associated phosphorylation of Rb proteins, we reasoned that lack of Ecd may promote continued association of Rb with E2Fs. To address this possibility in a physiological context, we examined the *in vivo* occupancy of Rb proteins on known E2F target promoters using the ChIP assay.

We carried out ChIP assays in synchronized cells after serum stimulation. As expected, the occupancy of Rb and p130 on B-myb and cdc2 promoters steadily declined as control MEFs transitioned out of G<sub>0</sub>/G<sub>1</sub> phase (Fig. 4D). In contrast, Rb and p130 continued to remain associated with E2F target promoters at 16 and 24h after serum addition in *Ecd*-null MEFs (Fig. 4D). These results showed that loss of Ecd protein results in the reduction of the dissociation of Rb proteins from E2Fs and suggested a role for Ecd in cell cycle progression.

***Ecd interacts with Rb family proteins***-While a number of potential mechanisms could be hypothesized to account for the role of Ecd in Rb-E2F dissociation implied by experiments presented above, a simple model would involve a potential interaction of Ecd with Rb proteins at or near the site where E2Fs bind.

To explore this model, we first tested the potential interaction of purified recombinant Ecd and Rb using a GST-pull down assay. Purified Ecd was pulled down by a GST-Rb fusion protein that incorporated the A/B and C pocket domains (amino acid 379-928) (Fig. 5A). Consistent with this *in vitro* interaction, immunoprecipitation analysis showed that endogenous Ecd and Rb proteins form a complex in MEFs (Fig. 5B). Reciprocal experiment using Ecd antibodies to

immunoprecipitate the complexes did not co-immunoprecipitate Rb. Further analysis revealed that the currently available Ecd antibodies inhibit the Ecd-Rb interaction (data not shown).

As a next step, we examined the binding of Ecd with other Rb family proteins, p107 and p130 in comparison to Rb. Notably, the *in vitro* studies indicated that Ecd interacts with all Rb family proteins although its interaction with Rb and p130 appeared more robust (Fig. 5C). Analysis of a series of truncation mutants of Ecd showed that only the full-length Ecd (amino acid 1-644) and the fragment encompassing amino acids 150-644 interacted with Rb (Fig. 5D). Furthermore, GST-pull down analysis using MEF cell lysates showed that Ecd interacts with the hypophosphorylated form of Rb, similar to E2F1 (Fig. 5E). GST-pull down assay with various fragments of Rb showed that Ecd interacts with the same region of Rb as does E2F1 (Fig. 5F). These results demonstrate that Ecd can directly bind to Rb family proteins and that the region of Rb where Ecd binds likely overlaps with E2F interaction region.

**Ecd inhibits Rb - E2F1 binding-**The existence of an Ecd-Rb complex in cells and *in vitro* demonstration that Ecd interacts with the pocket domains of Rb raised the possibility that Ecd may play a role in dissociation of Rb from E2F. To test this idea, we first examined if Ecd can compete with E2F for binding to Rb. We assessed the effect of purified Ecd on Rb-E2F1 interaction using a pull down assay with GST-E2F1 and purified Rb and Ecd proteins. Notably, addition of increasing amounts of Ecd in Rb-E2F1 binding reactions led to a diminution of Rb-E2F1 binding (Fig. 5G). In contrast to full-length Ecd, a C-terminal fragment (amino acid 439-644) of Ecd that showed little binding to Rb has no effect on Rb-E2F1 binding (Fig. 5H). In a converse experiment to assess whether both Ecd and E2F1 bind to a shared region on Rb, we examined the effect of purified GST-E2F1 on *in vitro* Rb-Ecd interaction using a Nickel bead pull down of 6X histidine-tagged Ecd. The presence of increasing amounts of GST-E2F1 but not of GST reduced the Rb-Ecd binding (Fig. 5I). As expected, HPV16 E7 competed with Ecd for binding to Rb, which further supports the observation that Ecd binds to Rb through its pocket domain (data not shown). Finally, we assessed the effects of adding purified Ecd on Rb-E2F complexes isolated from cells.

Addition of increasing amounts of Ecd to cell lysates led to a disruption of endogenous Rb-E2F complexes when analyzed by Rb co-immunoprecipitation with a cocktail of anti-E2F1-3 antibody (Fig. 5J). Taken together, these results clearly demonstrate that Ecd competes with E2F for binding to hypophosphorylated Rb and can facilitate disruption of Rb-E2F complexes.

**HPV16 E7 overcomes Ecd-deletion induced proliferative block.** According to the model deduced from analyses presented above that Ecd promotes Rb-E2F dissociation during cell cycle progression, we predicted that the proliferative block imposed by Ecd deletion will be reversed if the stabilized Rb-E2F complex can be forced to dissociate. Viral oncoproteins such as HPV16 E7 bind to pocket domains of Rb family proteins and induce their dissociation from E2F proteins (26). Indeed, expression of HPV16 E7 has been shown to rescue the impaired proliferation due to defects in Rb-E2F pathway in CDK2 and CDK4 double-knockout MEFs (27). Therefore, we stably expressed HPV16 E7 in *Ecd*<sup>lox/lox</sup> MEFs using retroviral infection (Fig. 6A) and subsequently infected the cells with Cre adenovirus. Cre-mediated deletion of Ecd was confirmed by immunoblotting (Fig. 6B). Notably, E7-expressing MEFs did not undergo a proliferative block despite Cre-mediated Ecd deletion (Fig. 6C). These analyses functionally validate the model that Ecd plays a role in cell cycle progression by promoting the dissociation of Rb-E2F complexes.

## DISCUSSION

Dissociation of Rb-E2F complexes is widely viewed as a critical event in G<sub>1</sub>-S cell cycle transition and the basic components and regulators of this pathway are commonly altered in a variety of human cancers (28-32). This pathway is also a prominent target of viral oncogenes implicated in human cancer (33-35). Further mechanistic insights into control of Rb-E2F dissociation during cell cycle progression are therefore of substantial interest in cell, developmental and cancer biology. Here, we identify the little understood Ecd protein, product of the mammalian orthologue of *Drosophila ecdysoneless* gene, as a novel cell cycle regulator and demonstrate that Ecd functions by interacting with Rb and facilitating Rb-E2F dissociation during cell cycle progression.

Our conclusion that Ecd plays a role in cell cycle progression is based on clear evidence from cell cycle analyses using mouse embryonic fibroblasts in which floxed Ecd was conditionally deleted using Cre recombinase. The proliferative arrest imposed by conditional deletion of Ecd was reversed by ectopically introduced human Ecd, thereby clearly establishing that loss of Ecd itself rather than any alteration of neighboring gene products was responsible for the observed cell cycle phenotype. In addition, Ecd-deletion led to delay of G<sub>1</sub>-S cell cycle progression. This represents the first formal demonstration of a role for Ecd in cell cycle progression.

While a number of mechanisms could be envisioned for a role of Ecd in cell cycle progression, analyses of biochemical events known to accompany the G<sub>1</sub>-S cell cycle progression strongly implicated its role in the Rb-E2F pathway. These included the substantial delay and overall reduction in the conversion of Rb from its hypophosphorylated to hyperphosphorylated state normally seen during G<sub>1</sub> to S transition. In addition, we observed a reduction in the total levels and activity of CDK2 while CDK4 levels and activity were unaltered. The latter observation prompted us to investigate if Ecd is required for cell cycle-associated E2F target gene expression. Biochemical analyses of E2F target gene products in Ecd-deleted vs. parental *Ecd*<sup>lox/lox</sup> MEFs firmly established that Ecd is required for cell cycle-associated E2F target gene expression and provide a logical basis for impaired cell cycle transit in cells deficient in Ecd.

A possible model of how Ecd could facilitate the E2F-dependent transcription, one for which our studies provide supportive evidence, is that Ecd facilitates Rb-E2F dissociation and thereby helps relieve E2F transcription factors from repression by Rb family proteins. Several key findings reported here support this model. First, Ecd deletion resulted in a drastic delay and overall reduction in Rb phosphorylation, a modification that has been established as a key link in Rb dissociation from E2F as cells progress through G<sub>1</sub>-S transition. Second, we showed, using ChIP assay, that more Rb as well as p130 was associated with E2F target promoters in *Ecd*-null MEFs. Third, CDK4 activity in *Ecd*-null cells is not altered, which suggests that Ecd does not function upstream of Rb phosphorylation. The decrease of

Rb hyperphosphorylation in *Ecd*-null cells is likely to reflect the reduced levels of E2F targets such as cdc2, CDK2, cyclin E, A and B, which are transcriptionally downregulated in *Ecd*-null cells. Fourth, we showed that Ecd directly interacts with Rb as well as with other Rb-family members. Fifth, we established that binding to Ecd prevents Rb from interacting with E2F1 and thereby facilitates the disruption of *in vitro* assembled as well as endogenous cellular Rb-E2F complexes. Finally, HPV16 E7, an Rb pocket-binding oncoprotein known to induce the dissociation of Rb-E2F complexes, reversed the cell cycle block imposed by Ecd deficiency, strongly arguing that the functional effects of Ecd deficiency on the cell cycle are due to impaired Rb-E2F dissociation. Collectively, the biochemical evidence together with functional effects of Ecd deficiency on E2F-mediated gene expression and cell cycle progression strongly support the model that Ecd plays a physiological role to facilitate the dissociation of Rb-family proteins from E2F transcription factors thereby helping to switch E2Fs to a transcriptional activation mode for cell cycle progression.

When we examined *in vivo* promoter occupancy by Rb proteins, we found that more Rb proteins were associated with the E2F target promoters in the *Ecd*-null MEFs. This demonstrates that the suppressed target gene expression in *Ecd*-deleted MEFs was caused by the association of the repressor Rb complex in these target gene promoters. Because Rb proteins associate with target promoters only through interaction with E2Fs, we next studied how Ecd affects Rb-E2F interaction in order to understand how Ecd functions in cell cycle regulation. Rb family proteins possess a 'pocket' domain through which it interacts with numerous cellular proteins and viral oncoproteins (36). Many proteins including E1A, E7, and TAg oncogenes have LxCxE sequence motif in order to interact with the Rb pocket domain (33). Competitive Rb interaction with cellular proteins is one of the mechanisms by which viral oncogenes dysregulate normal cell cycle. For example, E1A displaces E2F1 on Rb by competing for the binding to the pocket region which disturbs normal cell cycle. Here, we show that Ecd binds to Rb directly. The requirement of A/B pocket for Rb-Ecd interaction gave us the hint that Ecd may compete with E2F1

to bind to Rb because E2F1 also interacts with Rb through the same pocket region. *In vitro* and *in vivo* binding experiments have demonstrated that Ecd can displace E2F1 by competing with it for Rb binding. Given that Ecd inhibits Rb interaction with E2F1, Ecd may have a role in regulating E2F transcription by modulating Rb-E2F1 interaction.

In conclusion, we demonstrate that the

previously little understood but evolutionarily-conserved Ecd protein plays an important role in cell cycle progression. The cell cycle role of Ecd appears to involve its binding to hypophosphorylated Rb, thereby facilitating Rb-E2F dissociation and cell cycle progression. Thus, our studies identify Ecd as a novel component for physiological regulation of mammalian cell cycle.

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## FOOTNOTES

We thank Drs. Pradip Raychaudhuri (UIC, Chicago, IL) for GST-Rb and GST-E2F1 constructs, Amy Yee (Tufts University Medical School, Boston, MA) for GST-107 and CMV-p130 constructs, Karl Munger (Harvard University, Boston, MA) for GST-HPV16E7 construct and Izolda Papova (Northwestern University, Chicago, IL) for help in generating Ecd monoclonal antibody. This work was supported by the NIH Grant R01 CA96844-06 and CA94143, and Department of Defense grant W81XWH-07-1-0351 to V.B; NIH CA 87986, CA 105489, CA 99900, CA99163 and CA116552 to H.B. YZ is a Carol and Marvin Gollob Fellow. CBG is supported by the Department of Defense Postdoctoral Traineeship DAMD-17-03-1-0585. JHK is supported by a Korea Science and Engineering Foundation Grant (M06-2004-000-10547-0) and the Department of Defense Pre-doctoral Traineeship grant # W81XWH-08-1-0366. VB acknowledges the support of the Duckworth family through the Duckworth Family Chair for Breast Cancer Research. HB acknowledges the support from the Jean Ruggles-Romoser Chair for Cancer Research.

## FIGURE LEGENDS

**FIGURE 1. Ablation of Ecd causes a proliferation defect in MEFs.** *A*, growth curves of *Ecd*<sup>+/+</sup>, *Ecd*<sup>lox/lox</sup> and *Ecd*<sup>lox/lox/hEcd</sup> MEFs after control or adeno-Cre infection. Each time point shows the average cell number of triplicates. A representative experiment out of three is shown. *B*, for colony formation assay, cells infected with control or adeno-Cre viruses were grown for 10 days and stained with crystal violet. *C*, Ecd expression levels at different time points after adeno-Cre infection. Note that reconstituted control cells express both mouse (upper band) and human (lower band) Ecd, while only human Ecd is seen in adeno-Cre infected cells.

**FIGURE 2. Ecd disruption leads to G<sub>1</sub>-S delay in MEFs.** *A*, phase-contrast microscopic images of *Ecd*<sup>lox/lox</sup> MEFs 4 days after control or adeno-Cre virus infection. *B*, TUNEL assay of *Ecd*<sup>lox/lox</sup> MEFs 5 days after adeno-Cre infection. At least 500 cells were counted and percentage of apoptotic cells is shown in the graph. MEFs treated with adriamycin (Adr, 33 μM, 24 hrs) served as positive controls. *C*, BrdU staining of control or adeno-Cre virus infected *Ecd*<sup>lox/lox</sup> MEF after serum stimulation.

**FIGURE 3. Reduced Rb phosphorylation and CDK2 expression in Ecd-null MEFs.** *A*, and *B*, phosphorylation of Rb in asynchronous and synchronized/serum-stimulated MEFs. Open and solid arrowheads indicate hypo- and hyper-phosphorylated Rb proteins, respectively. *C*, expression of CDKs and CDK inhibitors in *Ecd*-null MEFs. *D* and *E*, *in vitro* kinase assay (KA) of extracts of serum-re-stimulated MEFs using anti-CDK4, or anti-CDK2 IPs. Anti-CDK4 or anti-CDK2 IP using 500 μg of *Ecd*<sup>lox/lox</sup> MEF extracts infected with control or adeno-Cre viruses were subjected to *in vitro* kinase assay. In one lane of *E*, purified Ecd protein was added to *Ecd*-null MEF extracts before IP.

**FIGURE 4. Reduced expression of cell cycle-related genes in Ecd-null MEFs.** *A*, immunoblotting for cell cycle regulatory proteins detected in the indicated serum stimulated *Ecd*<sup>lox/lox</sup> MEFs infected with control or adeno-Cre viruses. *B*, reconstitution with Flag-hEcd restores cyclin A expression in *Ecd*<sup>lox/lox</sup>

MEFs infected with adeno-Cre virus. The level of Ecd expression in control vector- or Flag-hEcd-expressing *Ecd*<sup>lox/lox</sup> MEFs after adeno-Cre infection is shown (left). *C*, *Ecd*<sup>lox/lox</sup> MEFs were infected with control or adeno-Cre viruses and 3 days after infection, cells were collected for real-time-PCR of the indicated genes. Error bars represent mean  $\pm$  SD from three independent PCR reactions. *D*, Wild type and *Ecd*-null MEFs were starved for 3 days and serum-stimulated. At different time points, samples were collected and used for ChIP assay with antibodies against Rb, and p130 followed by PCR amplification of the indicated E2F target promoters.

**FIGURE 5. Ecd interacts with Rb and inhibits Rb-E2F interaction.** *A*, *in vitro* binding assay with purified GST-Rb and Ecd. *B*, *in vivo* binding of Rb and Ecd in MEF extracts. Anti-Rb IPs from extracts of MEFs was immunoblotted with an anti-Ecd antibody. *C*, *in vitro* binding assay with purified Rb family proteins and full-length Ecd. *D*, GST or GST-Rb (amino acid 379-928) were incubated with lysates of 293T cells expressing the indicated truncated forms of Flag-Ecd and subjected to anti-Flag immunoblotting. *E*, Ecd preferentially binds to hypophosphorylated form of Rb. *In vitro* binding assay was performed with purified GST-Ecd (amino acid 150-644), GST-E2F1 and MEF cell extracts (a mixture of serum starved and asynchronous cells). Open and solid arrowheads indicate hypo- and hyperphosphorylated Rb forms, respectively. *F*, *in vitro* binding assay with purified GST-Rb (amino acid 379-928, 379-792 and 768-928) and Ecd. U2OS cell extracts expressing Flag-hEcd or E2F1 were incubated with purified GST-Rb proteins. *G*, purified GST-E2F1 (200 ng) and Rb (amino acid 379-928, 50 ng) were incubated in the absence or presence of Ecd (0.2, 0.5, 1, or 2  $\mu$ g). *H*, 1  $\mu$ g of full-length (amino acid 1-644) or N-terminally truncated (amino acid 439-644) Ecd proteins were used to compete with GST-E2F1 for binding to Rb. The purity of Ecd proteins is shown in right panel. *I*, purified Rb (50 ng) and Ecd-His (100 ng) were pulled down with Ni-beads in absence or presence of GST or GST-E2F1 (0.2 or 1  $\mu$ g). *J*, endogenous Rb/E2F complex can be dissociated by Ecd protein. Purified Ecd protein (0.2 or 2  $\mu$ g) was added to 100  $\mu$ g aliquots of T98G cell extracts prior to immunoprecipitation using a mixture of anti-E2F1-3 antibodies. E2F-associated Rb was detected by immunoblotting. Arrowheads in ponceau S stain figures indicate the purified GST/GST-fusion proteins

**FIGURE 6. HPV16 E7 overcomes Ecd-deletion induced proliferative block** *A*, RT-PCR of E7 expression in *Ecd*<sup>lox/lox</sup> MEFs infected with HPV16 E7. *B*, the expression level of Ecd in *Ecd*<sup>lox/lox</sup> MEFs expressing Flag-hEcd and HPV 16 E7 after adeno-Cre infection. Exogenously expressed Flag-hEcd migrates slightly slower than the endogenous mouse Ecd. *C*, colony formation assay of Flag-hEcd or E7 expressing *Ecd*<sup>lox/lox</sup> MEFs after control or adeno-Cre virus infection. Cells were stained with crystal violet and the solubilized dye absorbance was measured at 590 nm. Graph shows the relative rescue efficiency as compared to vector cells. Error bars represent mean  $\pm$  SD from three independent plates.

*Ecd*<sup>+/+</sup>

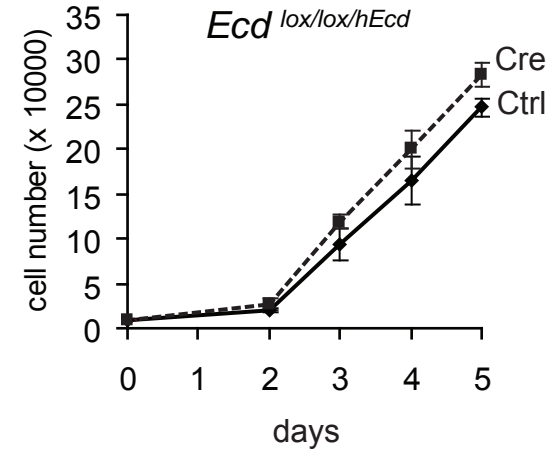
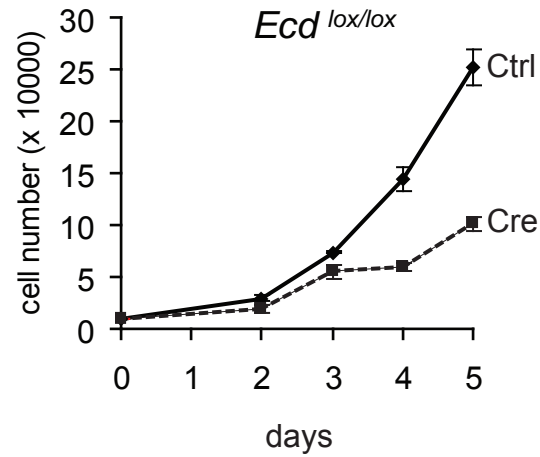
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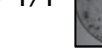

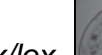
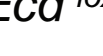
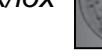


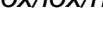
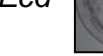
days

Ctrl

Cre

days	Ctrl (x 10000)	Cre (x 10000)
0	1.0	1.0
2	3.5	2.5
3	7.5	7.0
4	16.5	12.0
5	25.0	22.0

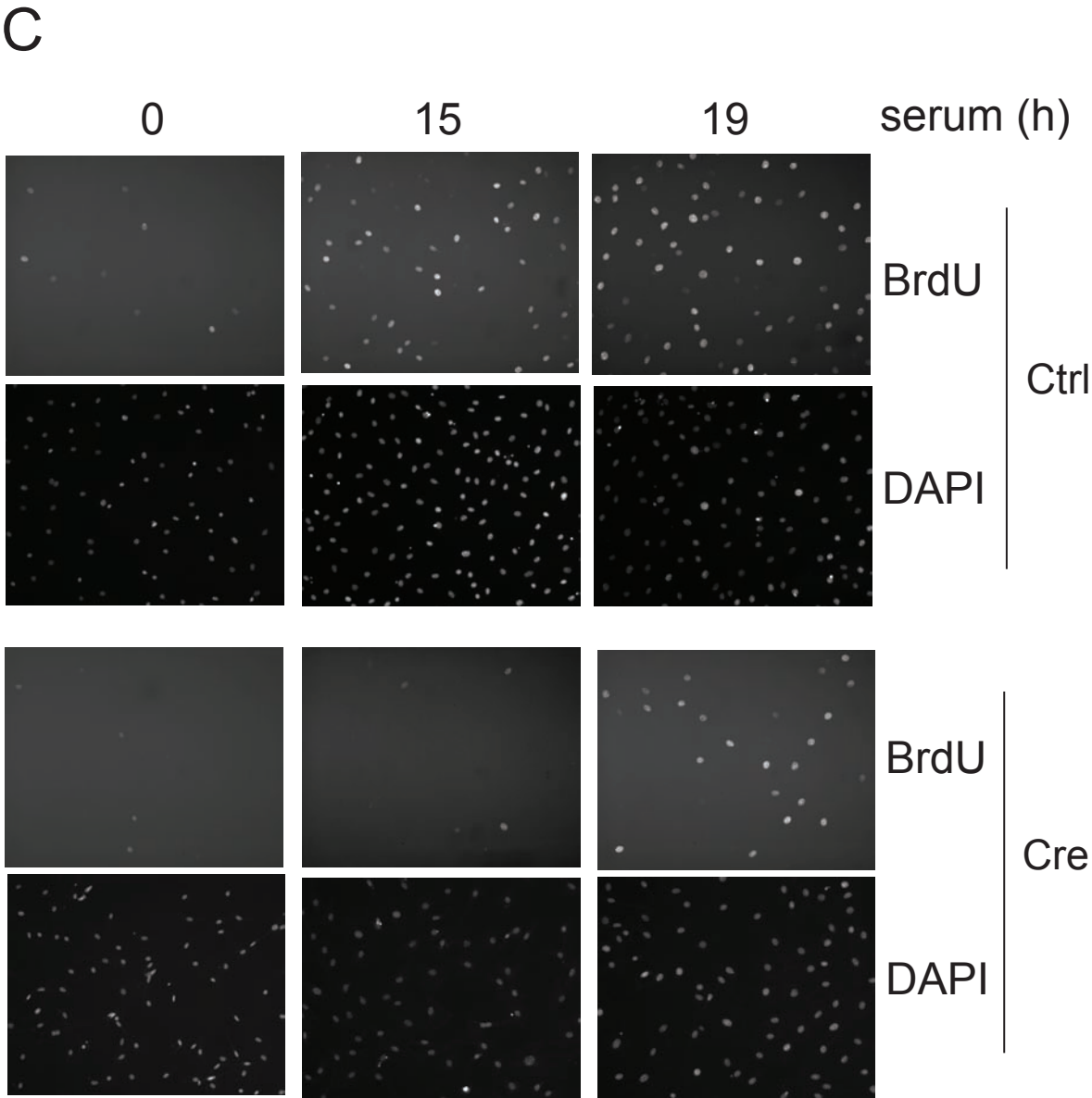
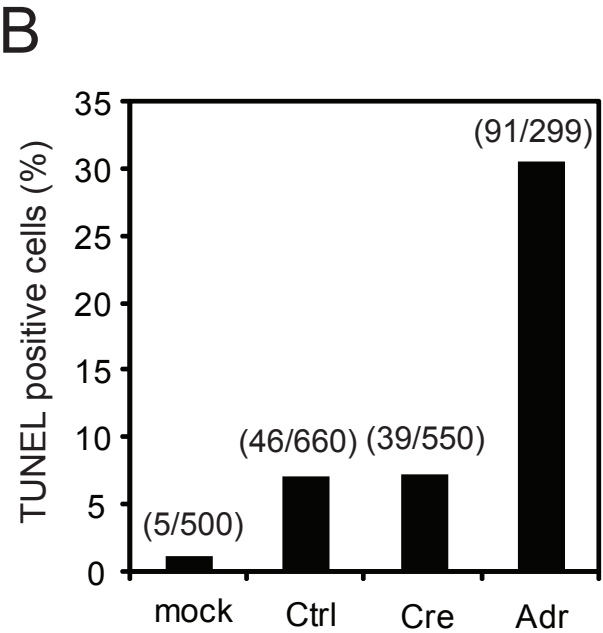
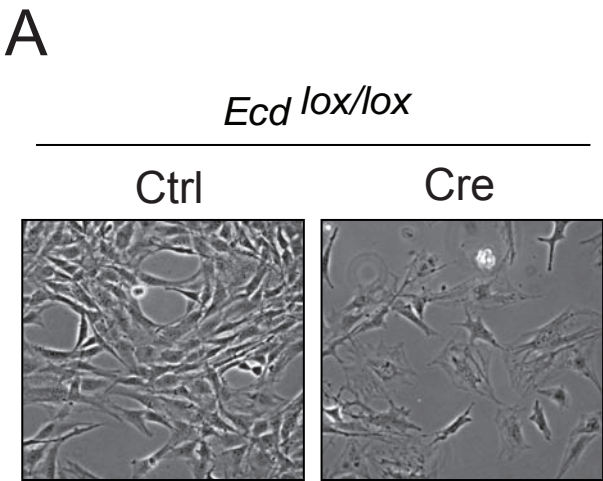


	Ctrl		Cre	
<i>Ecd</i> <sup>+/+</sup>				
<i>Ecd</i> <sup>lox/lox</sup>				
<i>Ecd</i> <sup>lox/lox/hEcd</sup>				

**C**

		Day 2		Day 3		Day 4		Day 5	
		Ctrl	Cre	Ctrl	Cre	Ctrl	Cre	Ctrl	Cre
<i>Ecd</i> <sup>+/+</sup>	Ecd								
	α-tubulin								
<i>Ecd</i> <sup>lox/lox</sup>	Ecd								
	α-tubulin								
<i>Ecd</i> <sup>lox/lox/hEcd</sup>	Ecd								
	α-tubulin								

Figure 2. Kim et.al.



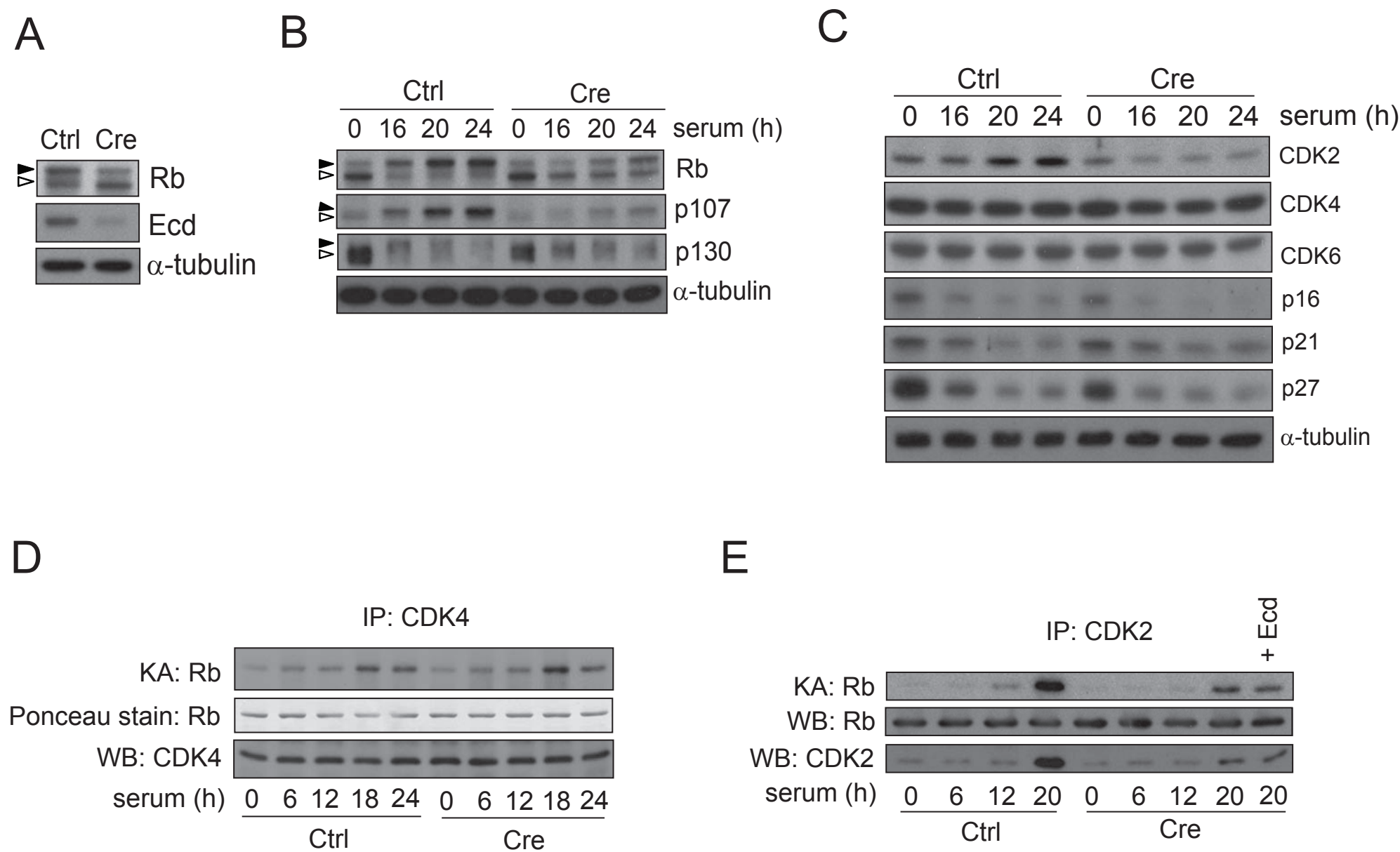
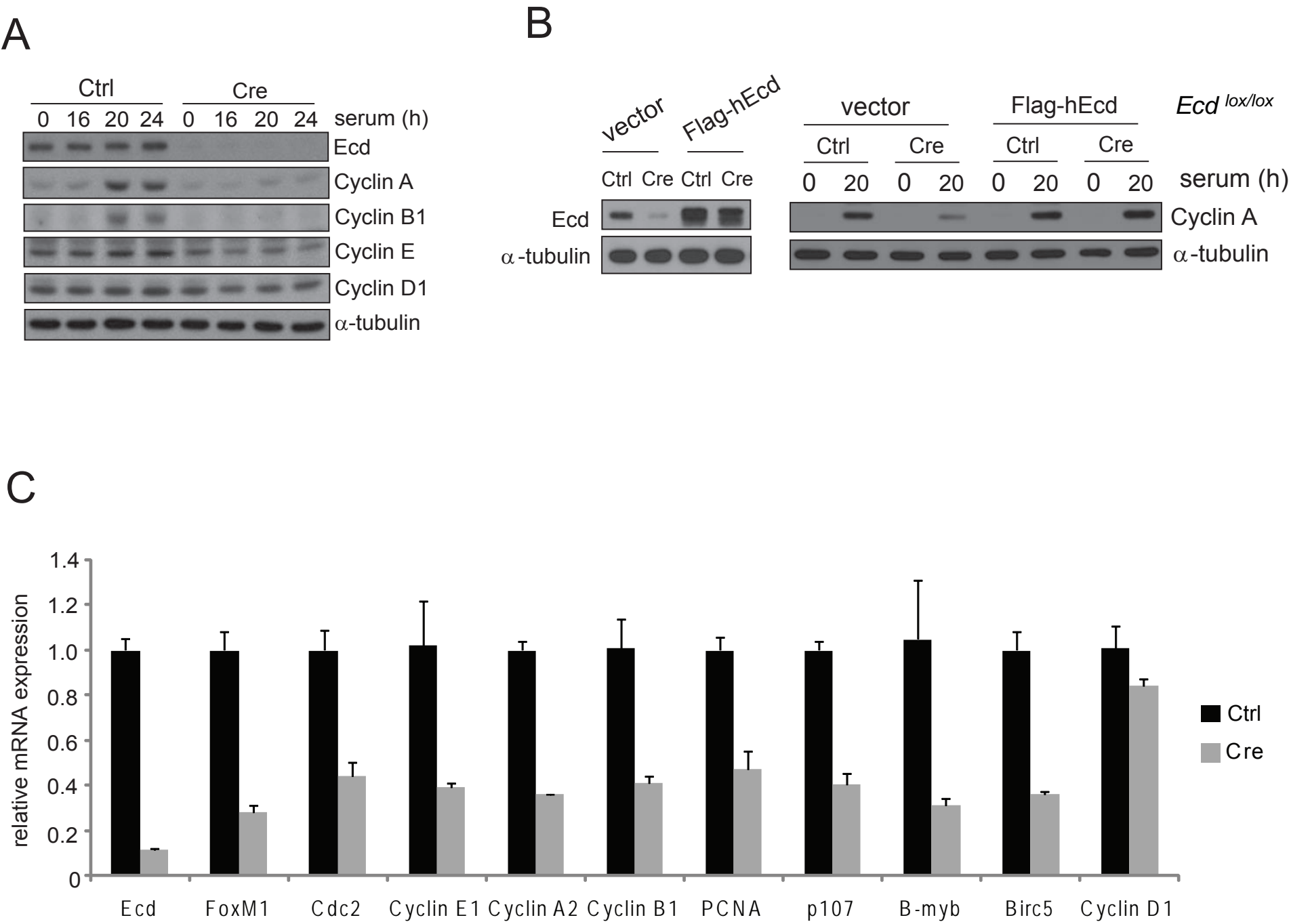


Figure 4. Kim et.al.



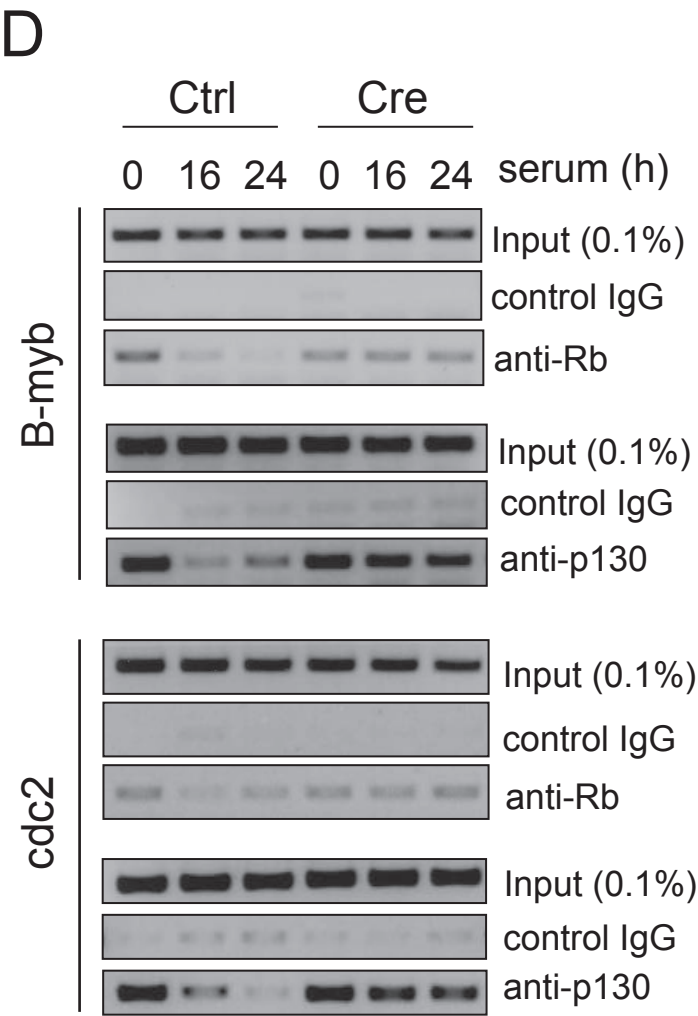


Figure 5. Kim et.al.

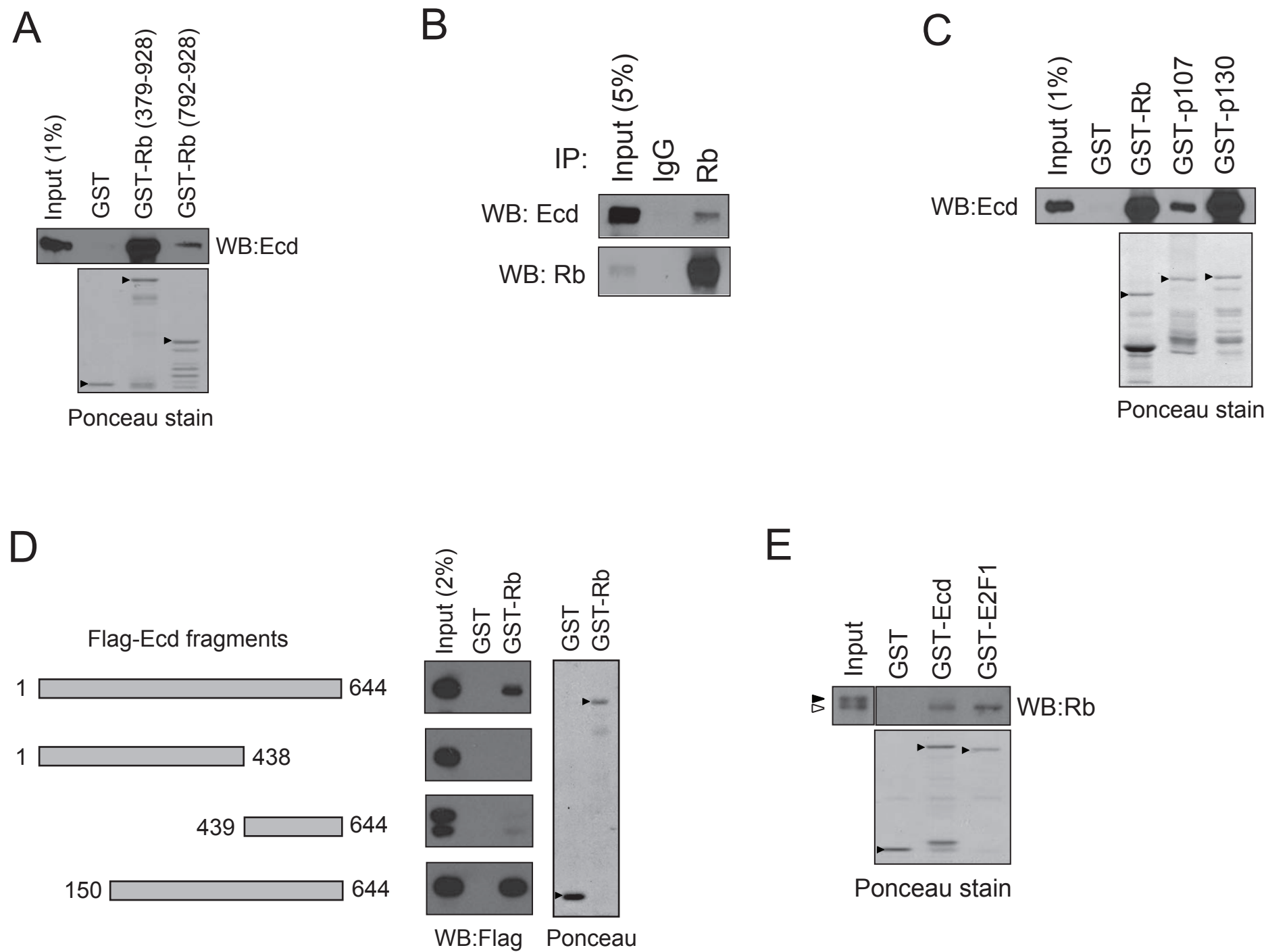
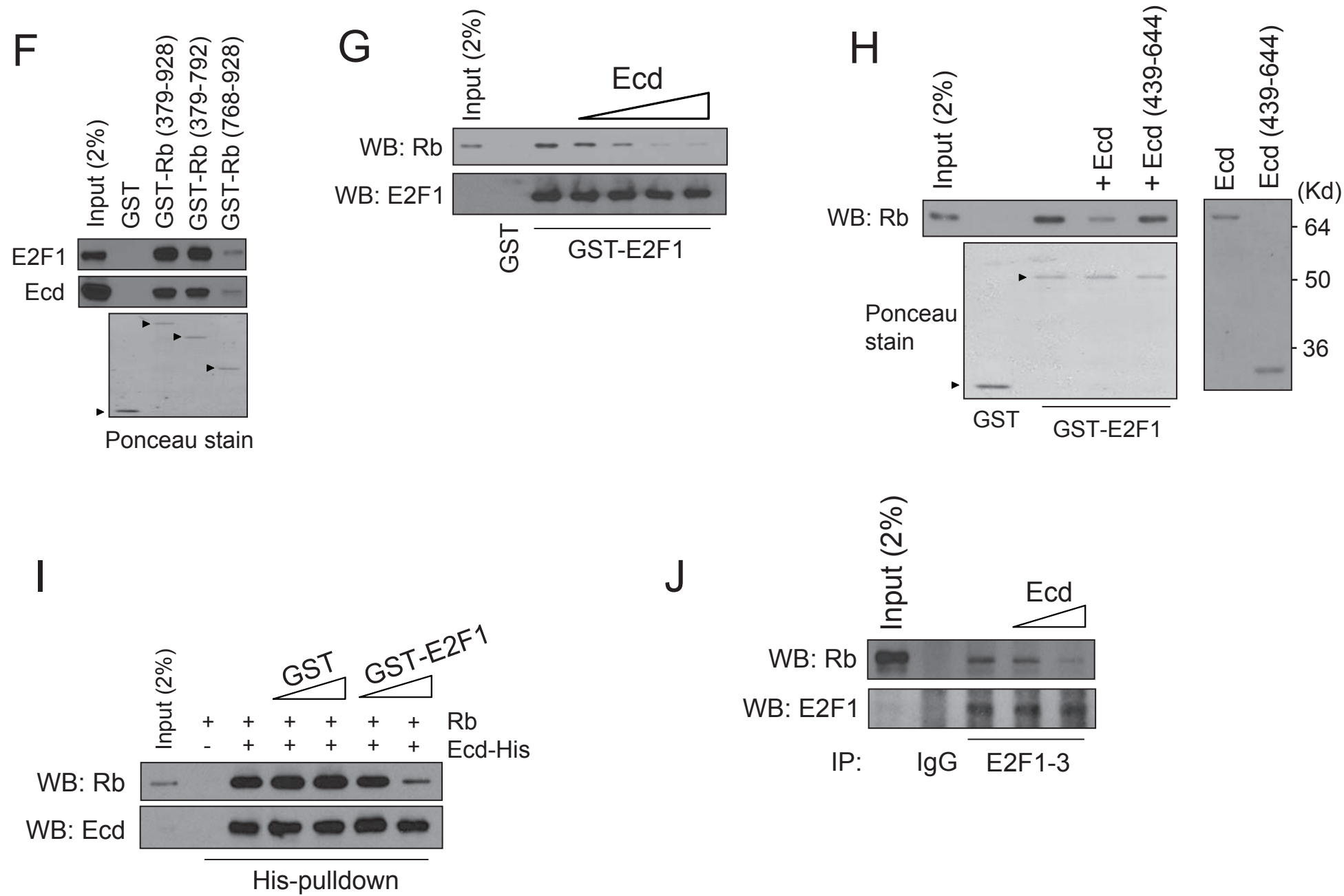
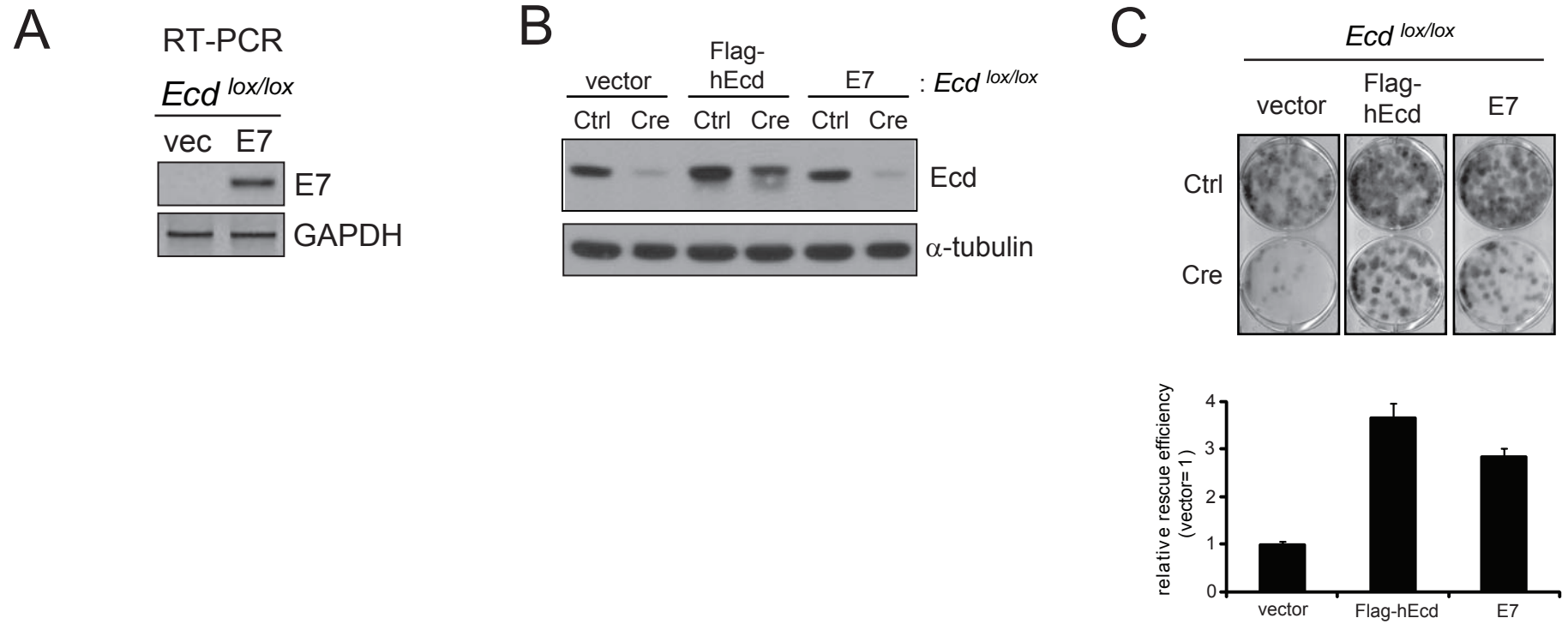




Figure 5. cont'd. Kim et.al.





# Biochemical characterization of human Ecdysoneless reveals a role in transcriptional regulation

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## Abstract

Ecdysoneless (Ecd) is an evolutionarily conserved protein and its function is essential for embryonic development in *Drosophila* and cell growth in yeast. However, its function has remained unknown until recently. Studies in yeast suggested a potential role of Ecd in transcription; however, Ecd lacks a DNA-binding domain. Using a GAL4-luciferase reporter assay and a GAL4 DNA-binding domain fusion with Ecd or its mutants, we present evidence that human Ecd has a transactivation activity in its C-terminal region. Importantly, further analyses using point mutants showed that a single amino acid change at either Asp-484 or Leu-489 essentially completely abolishes the transactivation activity of Ecd. We further demonstrate that Ecd interacts with p300, a histone acetyltransferase, and the coexpression of Ecd with p300 enhances the Ecd-mediated transactivation activity. Ecd localizes to both nucleus and cytoplasm and shuttles between the nucleus and cytoplasm; however, it exhibits strong nuclear export. Based on previous yeast studies and evidence provided here, we suggest that Ecd functions as a transcriptional regulator. Our results indicate an important function of human Ecd and provide a basis to explore the transcriptional partners of Ecd.

**Keywords:** coactivator; Ecdysoneless; hSGT1; transactivation.

## Introduction

The Ecdysoneless (Ecd) gene was named after the phenotype of a mutant *Drosophila* that has low levels of ecdysone, an insect steroid hormone responsible for normal embryogenesis, larval molting and metamorphosis. Dysregulation of ecdysone levels during embryonic development leads to the failure of normal development (Garen et al., 1977). Nearly three decades after the initial isolation of the mutant flies, the gene responsible for this mutation was identified (Gaziovva et al., 2004). Ecd gene shows a strong evolutionary conservation throughout eukaryotes from fission yeast to humans suggesting a conserved biochemical function.

Human Ecd was first isolated and named as hSGT1 (human suppressor GCR two) by a complementation assay study in *Saccharomyces cerevisiae* (*S. cerevisiae*). GCR1 is a core transcription factor that is involved in the expression of glycolytic genes in *S. cerevisiae*. A *gcr1* mutant showed a severe defect in glycolytic gene expression (Uemura et al., 1997). GCR2 is a GCR1-interacting protein and functions as a coactivator of GCR1 in glycolytic gene expression. The GCR2 gene was initially identified through the characterization of a novel mutation that affected glycolytic gene expression in *S. cerevisiae*. The *gcr2* mutant phenotype was similar to that of *gcr1* mutant and it was subsequently shown that GCR2 interacts with GCR1 and functions as a coactivator for GCR1-mediated glycolytic gene expression (Zeng et al., 1997). The complementation study of a *gcr2* mutant strain with human cDNA library was performed to identify human genes that can rescue the *gcr2* phenotype. One human cDNA that could reconstitute the GCR2 coactivator function in yeast was named hSGT1. Importantly, the hSGT1/hEcd complementation resulted in a recovery from the cell growth defect observed in the *gcr2* mutant apparently by substituting for the coactivator function of GCR2 through hEcd interaction with GCR1. The authors suggested that hSGT1 could be a functional analog of GCR2. Notably, there is no sequence similarity between hSGT1/hEcd and GCR2 (Sato et al., 1999).

A recent study in *Schizosaccharomyces pombe* (*S. pombe*) showed that *S. pombe* Ecd (named spSGT1 in the reported study) is required for cell survival and regulates gene expression involved in carbohydrate metabolism, amino acid metabolism and energy pathways (Kainou et al., 2006). In addition, we previously showed that hEcd interacts with and stabilizes p53 and its overexpression in mammalian cells increases the transcription of p53 target genes (Zhang et al., 2006).

Given the paucity of knowledge on the structure and function of this protein, we hypothesized that Ecd might play a role in transcriptional regulation based on several lines of evidence: (i) in *S. cerevisiae*, human Ecd (hEcd) is able to bind to GCR1 and act as a coactivator by substituting for GCR2 (Sato et al., 1999); (ii) in *S. pombe*, Ecd was shown to be important in cell survival and loss of its expression suggested a possible role as a transcription regulator (Kainou et al., 2006); (iii) hEcd binds to p53, and increases p53-mediated transcription (Zhang et al., 2006); and (iv) hEcd also binds to Rb and regulates Rb/E2F pathway (Kim et al., 2009). This reference is not listed in the reference list.

Here, using a GAL4 DNA-binding domain (GAL4-DBD) fusion protein of Ecd and GAL4-reporter luciferase assays, we demonstrate that Ecd has an intrinsic transactivation activity. Significantly, a point mutation in the C-terminal region completely abolished the transactivation activity of Ecd. Ecd binds to p300 and cooperates with p300 to increase the transactivation activity. Moreover, Ecd shuttles between the nucleus and cytoplasm, and its cytoplasmic localization depends on active CRM1-mediated nuclear export. These results suggest that mammalian Ecd might function as a transcriptional regulator and this function seems to be conserved through evolution.

## Results

### Human Ecd has an intrinsic transactivation activity

There is no identifiable DNA binding-domain on hEcd and no potential primary transcription factor for hEcd. Our previous studies showed that Ecd interacts with p53 and increases p53-mediated transcription (Zhang et al., 2006). However, Ecd might have a function in stabilizing p53 protein level rather than increasing p53 transcription via associating with p53 on the p53 target promoter because chromatin immunoprecipitation experiments failed to show that Ecd associate with p53 on the p21 promoter (data not shown). Regarding the role of Ecd in the Rb/Ecd pathway, Ecd is not likely to associate with E2F target promoter through the interaction with Rb because (i) Rb can associate with the E2F target promoter only through the interaction with the E2F transcription factor and (ii) Ecd competes with E2F for Rb binding. Chromatin immunoprecipitation experiments also failed to show that Ecd associates with E2F target gene promoters, such as B-myb, cdc2 and cyclin B1 (data not shown).

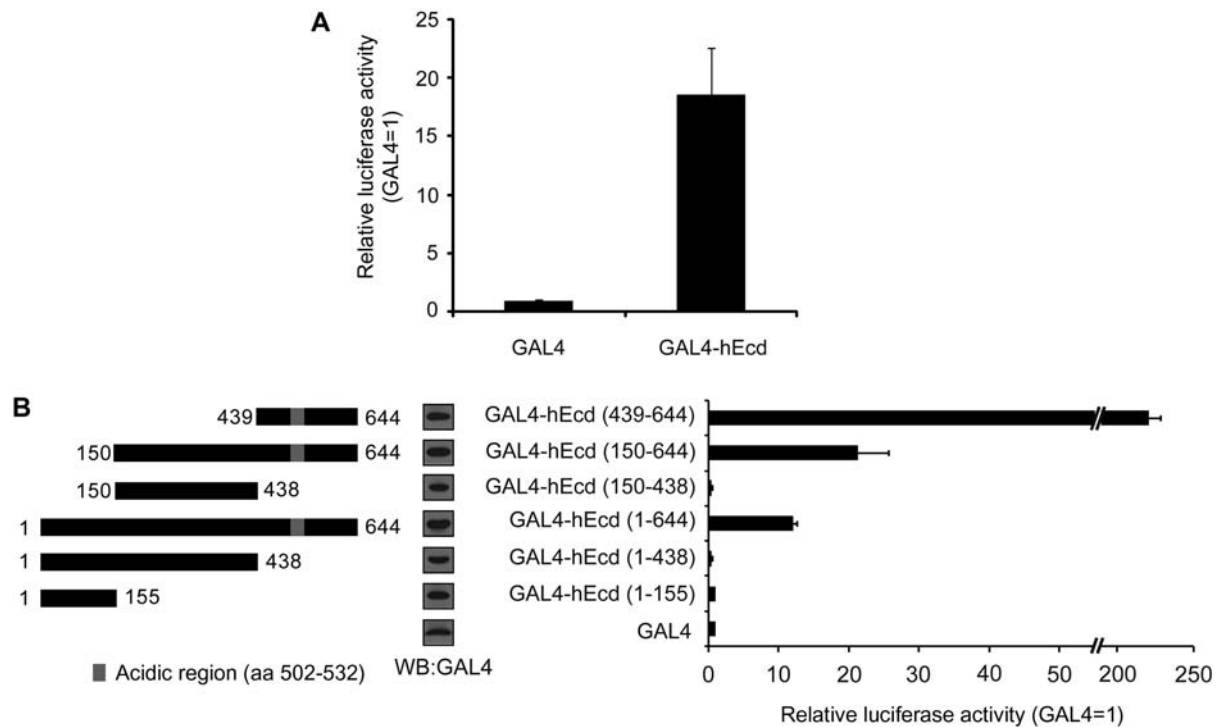
Therefore, to examine whether Ecd has a transactivation activity, a GAL4-reporter assay was performed. The GAL4-reporter assay is commonly used to study transactivation functions of transcription factors or transcriptional coactivators (Kim et al., 2003; Bratton et al., 2009). Full-length Ecd was fused to GAL4-DBD (DNA-binding domain) and introduced into cells together with pG5/luciferase reporter containing 5× GAL4 DNA-binding element upstream of the luciferase gene. Expression of the luciferase reporter gene is regulated by transcription factors that associate with the

upstream GAL4 DNA-binding element. To examine if mammalian Ecd has a transactivation activity, GAL4-DBD-fused full-length hEcd was introduced into U2OS cells together with pG5/luciferase reporter plasmid (SV40-driven *Renilla* luciferase cloned within the GAL4-DBD vector served as a transfection efficiency control). As shown in Figure 1A, hEcd was able to enhance the luciferase gene expression compared to GAL4-DBD control, which suggests that mammalian Ecd has an intrinsic transactivation activity. These results are consistent with the possible transactivation function of Ecd previously shown in yeast (Sato et al., 1999).

### The transactivation activity of Ecd resides in the C-terminal region

Given the lack of identifiable domains in Ecd, five GAL4-fused deletion mutants (aa 1–155, 1–438, 150–438, 150–644 and 439–644) were generated based on the secondary structure prediction (Jpred software, <http://www.compbio.dundee.ac.uk/jpred/>). To define the region(s) in Ecd required for transactivation, these GAL4-fused truncated forms of hEcd were then tested for transactivation activity. Notably, the C-terminal region (aa 439–644) was required for strong transactivation activity. A small fragment of the C-terminus (aa 439–644) showed even stronger activity when compared with the full-length hEcd (Figure 1B). The N-terminal region (aa 1–438) of Ecd protein might have an inhibitory function for the autonomous transactivation activity of its C-terminal region by changing the protein intramolecular folding structure like other transcription factors (Lillicrop et al., 1994; Dennig et al., 1996; Zhao et al., 2002; Park et al., 2008). As shown in Figure 2, the C-terminal region (aa 439–644) is well conserved in other species. Notably, Ecd contains a putative acidic region (aa 502–532) located in the C-terminal fragment (Figure 2). Acidic domains in other proteins are often associated with a transactivation function (Ma and Ptashne, 1987). To examine if the acidic region is involved in the transactivation activity, several point mutants (D510R, D512R or D520P) of the acidic region were generated in the context of the hEcd C-terminal fragment and analyzed using the GAL4-reporter assay. The mutations D510R or D512R in aa 439–644 fragment increased Ecd transactivation by twofold (Figure 3A). A shorter fragment (aa 467–644) exhibits transactivation activity comparable with that of the 439–644 fragment (Figure 3B); mutation of D520P in this shorter fragment also increased the transactivation activity (Figure 3B). These mutational analyses suggest that the structural changes in this acidic region introduced by single amino acid substitutions affect the transactivation activity of hEcd protein.

Given that the mutations in the acidic region affects transactivation activity, further mutational analyses in the upstream sequences of the acidic region were tested in the GAL4-reporter assay. The I481A mutation led to approximately 50% decrease in transactivation activity compared to wild type, whereas the S487A mutation had no effect (Figure 3C). Interestingly, a single point mutation D484A in the



**Figure 1** Mammalian Ecd has an intrinsic transactivation activity.

(A) To test if mammalian Ecd has transactivation activity, GAL4-fused human Ecd was transfected into U2OS cells together with GAL4-reporter plasmid. Twenty hours after transfection, cells were lysed and luciferase activities were measured as described in the materials and methods section. (B) Five deletion mutants of GAL4-hEcd were tested by GAL4-reporter assay. GAL4-fused hEcd constructs and pG5/luciferase plasmid were co-transfected into U2OS cells and luciferase activities were measured 20 h after transfection using the Dual-Luciferase Reporter Assay System. Luciferase activities were normalized with control *Renilla* luciferase values and relative activity is shown compared with GAL4 control. Error bars represent standard deviation from the means of triplicate samples. Cell lysates from the luciferase activity measurement were subjected to Western blotting using anti-GAL4 antibody to show the expression levels of different GAL4-hEcd forms.

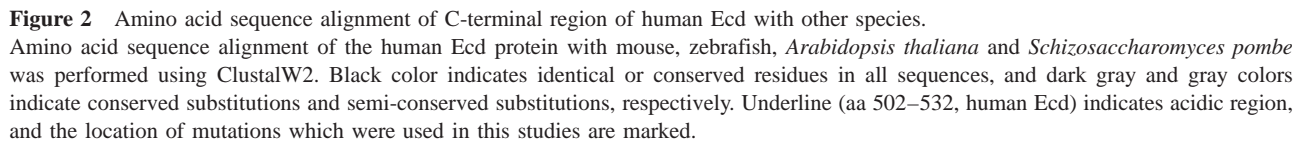
context of 439–644 fragment essentially abrogated all the transactivation activity (Figure 3C). Moreover, another mutation L489A clearly eliminated the transactivation activity of hEcd (Figure 3C). The same effect was confirmed in full-length hEcd when a single point mutation (D484F) was introduced (Figure 3D). Thus, Asp-484 and Leu-489 seem to be critical residues for hEcd transactivation activity and substitution of either of these amino acids can disrupt the transactivation activity of hEcd.

The C-terminus part of the acidic region is also well conserved between species (Figure 2). Therefore, we tested two C-terminally truncated forms (aa 1–610 and 1–539) of hEcd fragment for transactivation function. The fragment aa 1–610 demonstrated similar transactivation activity as full-length Ecd (Figure 3E, left), which suggests that deletion of aa 611–644 does not affect transactivation activity. However, the truncated fragment that spans aa 1–539 was defective in transactivation (Figure 3E, right). The acidic region (aa 502–532) might play a role in exerting potential transactivation activity together with proper functional structure of adjacent sequences.

### p300, a global transcriptional coactivator interacts with Ecd and increases the transactivation activity of Ecd

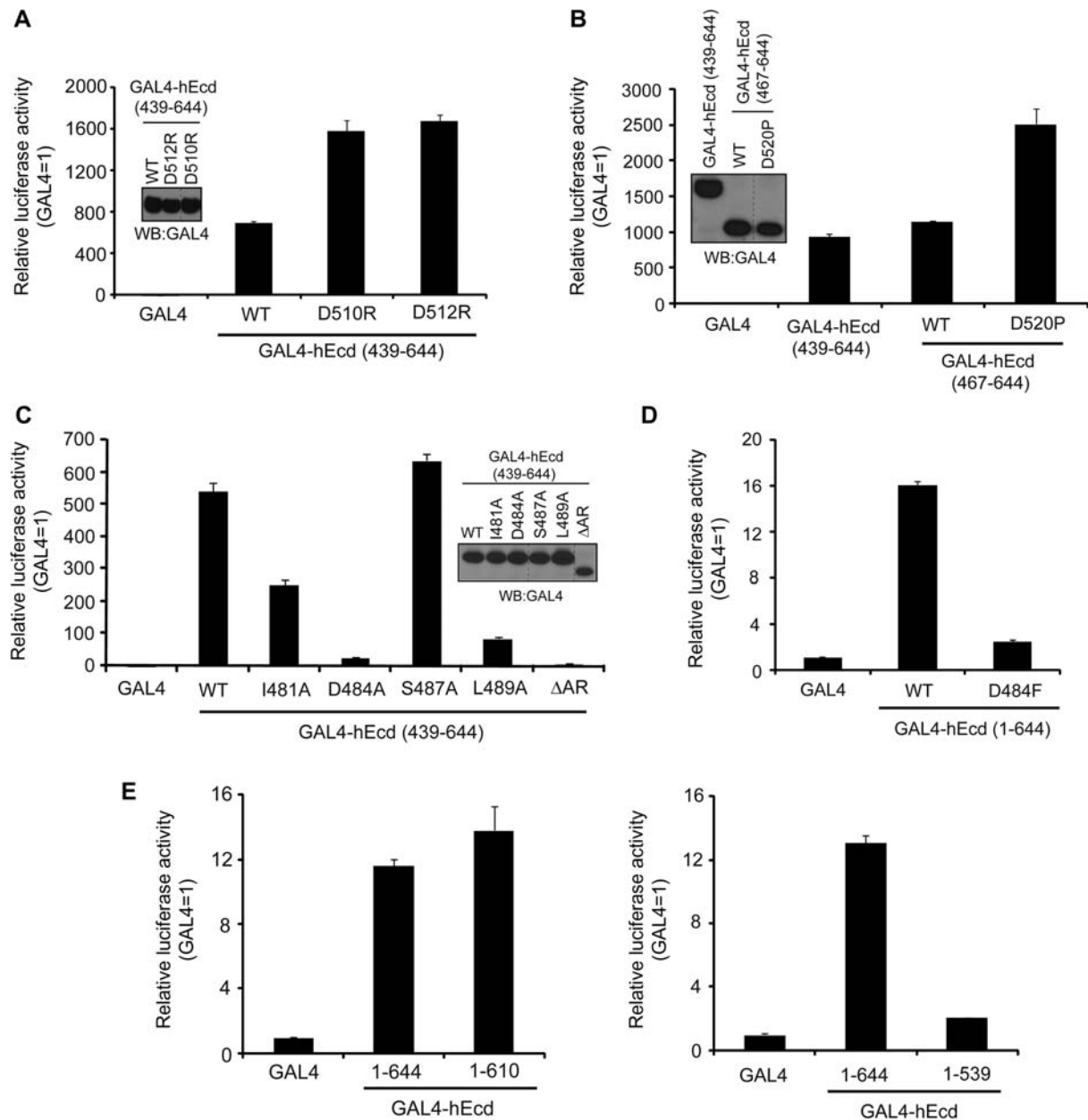
The p300 protein is a global transcriptional coactivator which binds to several transcription factors and associates with other coactivators, thereby increasing the expression of target genes. This function involves the recruitment of the basal transcription machinery to the promoter and promotion of acetylation of transcription factors and the histone proteins (Imhof et al., 1997; Blobel, 2000; Goodman and Smolik, 2000; Sterner and Berger, 2000; Chan and La Thangue, 2001; Vo and Goodman, 2001). To test whether p300 plays a role in Ecd-mediated transactivation, p300 was coexpressed with GAL4-hEcd in the GAL4-reporter assay. Transfection of increasing amounts of GAL4-hEcd plasmid led to a dose-dependent transactivation activity in both the absence and presence of p300. Notably, coexpression of p300 increased the transactivation activity of GAL4-hEcd compared with that of control plasmid expression (Figure 4A). GFP plasmid was co-transfected in all samples to verify comparable trans-





Given the effect of p300 on Ecd-mediated transactivation, we examined whether Ecd directly interacts with p300 by performing immunoprecipitation followed by Western blotting. For immunoprecipitation, Flag-hEcd was coexpressed in 293T cells in the absence or presence of HA-tagged p300. Cell extracts were immunoprecipitated using anti-HA antibody and Flag-hEcd that is associated with p300 was detected by Western blotting using anti-Flag antibody. A clear association of Flag-hEcd with p300 was detected in the immunoprecipitates from HA-p300 coexpressed cell extracts (Figure 4D). This interaction was also confirmed by the

One of the criteria for a protein to be a transcription factor or a transcriptional regulator is its ability to localize to the nucleus. To examine the subcellular localization of hEcd, Flag or GFP-fused full-length hEcd proteins were expressed in U2OS cells. The majority of Flag-hEcd and GFP-hEcd expressions were found in the cytoplasm (Figure 5A). However, biochemical fractionation of nuclear and cytoplasmic extracts showed the presence of nuclear as well as cytoplasmic Ecd (Figure 5B). The integrity of the fractionation was confirmed by immunoblotting against PARP and  $\alpha$ -tubulin, which are markers for nuclear and cytoplasmic extracts, respectively. These results suggest that Ecd localizes in both the nucleus and cytoplasm and the localization can be



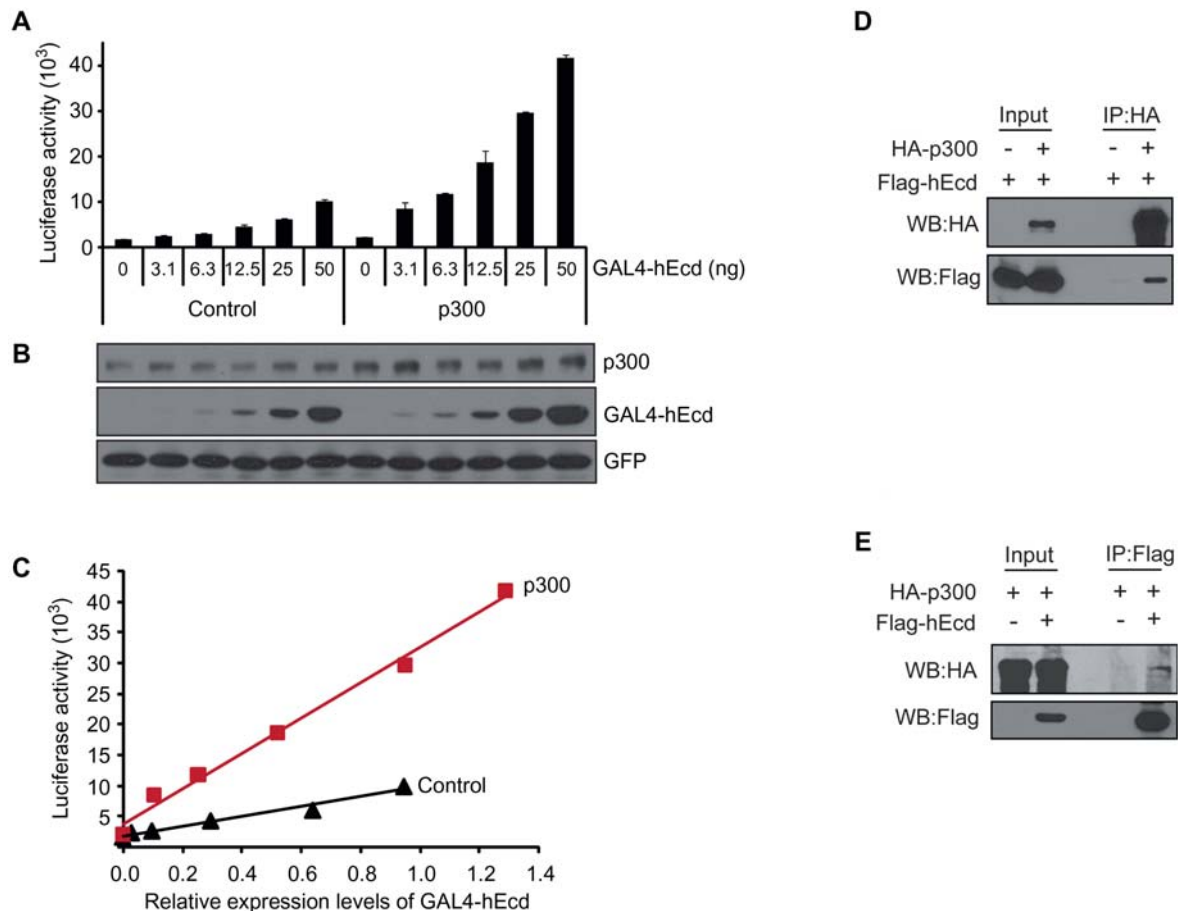
**Figure 3** Mutational analyses of human Ecd in a transactivation assay.

(A) Two point mutations (D510R or D512R) were introduced in GAL4-hEcd (aa 439–644) and these plasmids were transfected into U2OS cells. Relative activities are shown compared with GAL4 control. (B) D520P mutation was introduced in GAL4-hEcd (aa 467–644) and tested for transactivation activity. (C) Several indicated mutants (I481A, D484A, S487A, L489A and ΔAR) in GAL4-hEcd (aa 439–644) were tested for their effect on transactivation activity. (D) Full-length wild type and D484F mutant GAL4-hEcd were tested for transactivation activity. (E) Two C-terminally truncated mutants of GAL4-hEcd (aa 1–539 and 1–610) were used for reporter gene assay. All luciferase assays were performed as described in the materials and methods section. The relative activities of GAL4-hEcd are shown compared with GAL4 control. Error bars represent standard deviation from the means of triplicate samples. Western blotting using anti-GAL4 antibody shows relative expression levels of GAL4-hEcd.

dynamic. To test whether Ecd shuttles between the nucleus and cytoplasm, Flag- or GFP-tagged hEcd were expressed in U2OS cells and the localization of Ecd was detected with or without treatment with leptomycin B (LMB), a CRM1-mediated nuclear export inhibitor. A significant amount of Ecd accumulated in the nucleus upon LMB-induced inhibition of

nuclear export (Figure 5A). These results suggest that Ecd shuttles between the nucleus and cytoplasm, and that Ecd can be rapidly exported to the cytoplasm.

To further characterize the nuclear localization and export of Ecd, a chimera of Flag-hEcd fused with SV40 large T antigen nuclear localization signal (NLS; PKKKRKV) was



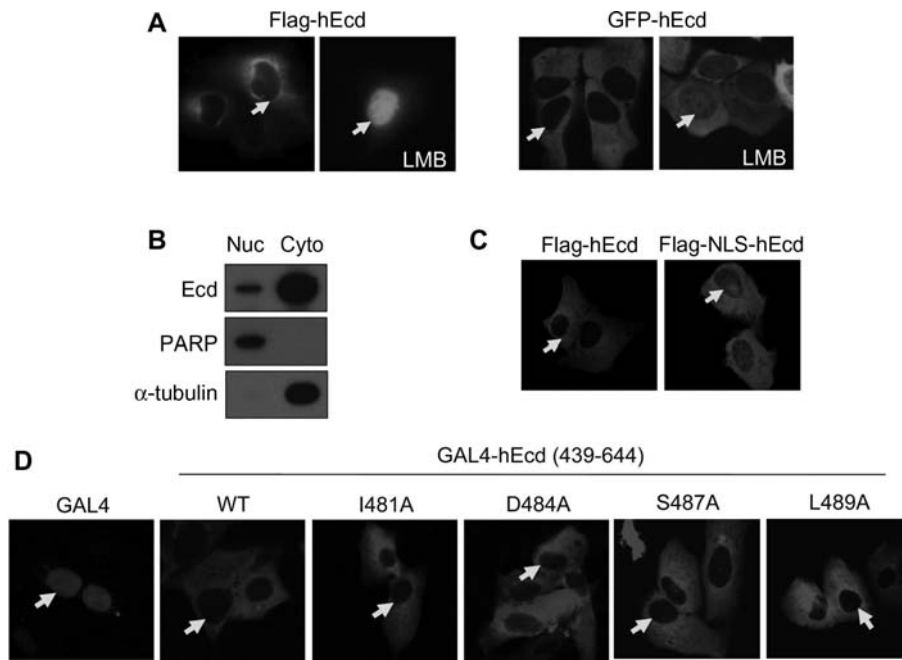
**Figure 4** Coexpression of p300 with Ecd increases Ecd transactivation activity and hEcd interacts with p300.

(A) 293T cells seeded in 24-well plates were transfected with different amounts of GAL4 control plasmid or full-length GAL4-hEcd plasmid together with 100 ng of pG5/luciferase, 50 ng of GFP (as a transfection control) and 100 ng of HA-p300 plasmids. Total luciferase activity was measured from cell lysates of transfected cells, which were cultured for additional 24 h post-transfection. (B) The expression of GAL4-hEcd and p300 is shown by Western blotting using GAL4 and p300 antibody, respectively. GFP expression is shown as a transfection control. Immunoblotting with p300 antibody detects both endogenous and exogenous p300. (C) The graph represents the comparison of luciferase activity versus the expression level of GAL4-hEcd in the absence or presence of p300 coexpression. The expression of GAL4-hEcd and GFP was quantified using ImageJ software (●●name of manufacturer, city, country?●●) and the relative expression level of GAL4-hEcd (signal ratio of GAL4-hEcd to GFP) is shown. (D) 293T cells were transfected with Flag-hEcd plasmid along with or without HA-p300 plasmid. Cell extracts were immunoprecipitated using anti-HA antibody and Flag-hEcd associated with HA-p300 was detected by Western blotting using anti-Flag antibody. (E) Extracts of 293T cells expressing HA-p300 together with or without Flag-hEcd were immunoprecipitated with anti-Flag antibody followed by Western blotting using anti-HA antibody.

generated. As shown in Figure 5C, although there was a moderate increase in the nuclear Ecd pool, a majority of Flag-NLS-hEcd was again localized in the cytoplasm. Additionally, we analyzed the subcellular localization of GAL4-hEcd. Because GAL4-DBD possesses an intrinsic NLS and GAL4-hEcd showed transactivation activity in the GAL4-reporter assay, we expected GAL4-hEcd to localize to the nucleus. As expected, GAL4 expression alone was in the nucleus; however, the GAL4-hEcd (aa 439–644) chimera was predominantly present in the cytoplasm although it showed very strong transactivation activity in the GAL4-reporter assay (Figure 5D). Next, several mutants of GAL4-

hEcd (aa 439–644) containing single amino acid substitution (I481A, D484A, S487A and L489A) that showed different transactivation activity were tested for localization. Notably, none of these mutants showed any difference in the subcellular localization (Figure 5D), suggesting that the transactivation defective phenotypes of D484A and L489A mutation were not as a result of different subcellular localization of these mutant forms of Ecd. These results support the concept that transient nuclear localization is sufficient for Ecd to exhibit transactivation activity. These results also suggest that Ecd has a strong nuclear export signal (NES) and its nuclear localization is transient.





**Figure 5** Ecd shuttles between nucleus and cytoplasm.

(A) Flag-hEcd was transfected into U2OS cells. Twenty-four hours after transfection, the localization of Flag-hEcd was shown by fluorescence immunostaining using anti-Flag antibody. GFP-hEcd was also analyzed 24 h after transfection. Twenty hours after transfection, cells were treated with 5 ng/ml of leptomycin B (LMB) for 4 h. (B) Biochemical fractionation of U2OS cell extract is shown. Nuclear (Nuc) and cytoplasmic (Cyto) extracts were prepared as described in the materials and methods section. To confirm purity of fractionation, extracts were probed against PARP (nuclear protein marker) and  $\alpha$ -tubulin (cytoplasmic protein marker). (C) Chimera of Flag-hEcd fused with SV40 large T antigen NLS (PKKKRKV) was generated by inserting the NLS motif between Flag and hEcd sequences. Flag-NLS-hEcd plasmid was transfected into U2OS cells. Twenty hours after transfection, Flag-NLS hEcd signal was visualized by immunostaining using anti-Flag antibody. (D) Wild type and mutant (I481A, D484A, S487A and L489A) of GAL4-hEcd (aa 439–644) plasmids were transfected into U2OS cells, and 20 h after transfection GAL4-hEcd signal was visualized by immunostaining using anti-GAL4 antibody. Fluorescence immunostaining was performed as described in the materials and methods section. Arrowheads indicate nucleus-cytoplasm boundary.

### The deletion of aa 481–497 region disrupts the cytoplasmic localization of Ecd

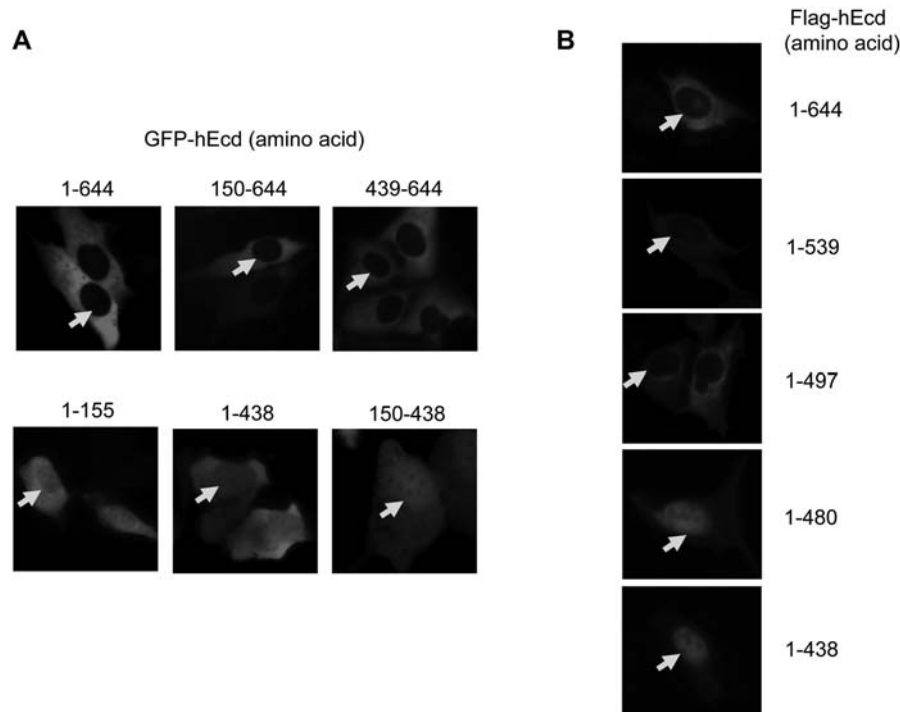
To characterize the regions of Ecd required for predominant cytoplasmic localization, five GFP-tagged deletion constructs (aa 1–155, 1–438, 150–438, 150–644 and 439–644) were transiently expressed in U2OS cells and the localization of these different fragments was examined. Although the full-length Ecd or its aa 150–644 and 439–644 fragments were mostly detected in cytoplasm, three fragments of Ecd (aa 1–155, 1–438 and 150–438) were detected in both the nucleus and cytoplasm (Figure 6A). These results suggest that the aa 439–644 region is responsible for the cytoplasmic localization of Ecd.

To further examine the subcellular localization, we analyzed additional truncated hEcd fragments. A series of Flag-tagged C-terminal truncated fragments (aa 1–539, 1–497, 1–480 and 1–438) were expressed in U2OS cells and tested for the localization by immunostaining using an anti-Flag antibody. The aa 1–539 and 1–497 fragments localized in the cytoplasm similar to the full-length Ecd; however, aa 1–480 fragment was localized in the nucleus (Figure 6B). This observation leads us to conclude that the short segment

481–497 seems necessary for Ecd cytoplasmic localization. Although this region does not have a canonical nuclear export sequence (la Cour et al., 2003), it might function as a potential NES. It is also possible that the deletion of 481–497 region might disrupt the motif for the cytoplasmic localization that resides in the vicinity of this segment. Taken together, these results demonstrate that Ecd localizes to both nucleus and cytoplasm and shuttles between the nucleus and cytoplasm with a rapid nuclear export.

### Discussion

The Ecd protein family is highly conserved from yeast to man, indicative of a conserved function of sequences retained through evolution. At present, however, no specific structural domains or motifs linked to Ecd function have been identified in any species. The first potential role of Ecd was suggested in yeast where hEcd (named hSGT1) was able to substitute for the coactivator function of GCR2 in *S. cerevisiae* (Sato et al., 1999) and deletion of Ecd in *S. pombe* (spSGT1) showed that Ecd is important for cell growth and regulates the expression of many genes involved



**Figure 6** The deletion of aa 481–497 region disrupts the cytoplasmic localization of Ecd.

(A) GFP-tagged full-length or truncated forms of hEcd (aa 150–644, 439–644, 1–155, 1–438 and 150–438) were transfected into U2OS cells. After 24 h post-transfection, GFP signal was observed under fluorescent microscope. (B) Flag-tagged full-length or C-terminal truncated forms of hEcd (aa 1–539, 1–497, 1–480 and 1–438) were transfected into U2OS cells. Twenty four hours after transfection, Flag-hEcd signal was detected by fluorescence immunostaining using anti-Flag antibody as described in the materials and methods section. Arrowheads indicate nucleus-cytoplasm boundary.

in cellular processes including various metabolic pathways (Kainou et al., 2006). Furthermore, *S. pombe* Ecd appears to be localized to the nucleus and to play an important role in the transcription of various genes. These observations led us to explore whether hEcd possesses properties of a transcriptional regulator.

Results of a GAL4-reporter gene assay using GAL4-DBD-fusion of Ecd show that hEcd possesses transactivation activity. This is the first evidence that mammalian Ecd might play a role in transcriptional regulation. Owing to lack of known DNA-binding motif, Ecd is expected to function as a transcriptional regulator rather than a transcription factor, which is consistent with its ability to substitute for coactivator GCR2 in yeast. hEcd contains 644 amino acids. Several regions in Ecd are highly conserved from yeast to humans, whereas other regions are conserved among higher organisms (*Drosophila* and higher). Initial mutational analyses presented here show that the C-terminal region of Ecd is crucial for its function as a transcriptional regulator. By a series of mutational analysis, we showed that Asp-484 and Leu-489 are critical for transactivation, as each single point mutation was able to abolish the transactivation activity. We opted to examine this region considering its strict conservation (Asp-484) among different species.

At present, it is difficult to speculate how the point mutations in the acidic region (D510R, D512R or D520P)

increase transactivation activity because the structure of Ecd protein is not known. These point mutations in the acidic region might cause a slight structural change in activation domain, which might impart increased transactivation activity to these mutants. In our analyses, the two mutant fragments 1–610 and 1–539 showed different transactivation abilities even though both mutants contain the acidic activation region aa 502–532. Although not experimentally proven, we speculate that the 1–539 fragment might disrupt proper structure of activation domain owing to its close proximity to the acidic region. By contrast, deletion 611–644 did not change activity; this might be because the deletion is distant from the acidic region in the primary structure.

Further evidence to support the observation that Ecd functions as a transcriptional regulator is provided by its cooperativity with p300, a histone acetyltransferase (HAT) for its transcriptional function. It is well documented that transcriptional factors or other transcriptional regulators directly interact with HAT proteins, such as p300 to perform a role in transcriptional regulation. Using *in vivo* assays, we present evidence for direct interaction of Ecd with p300 and cooperatively of p300 to augment the transactivation activity of Ecd. These results support the notion that mammalian Ecd has the biochemical properties of a transcriptional regulator.

Ecd derives its name from *Drosophila* studies where Ecd mutants show the defective phenotypes in embryo develop-

**Table 1** Primer sequences used in cloning experiments.

Plasmid (amino acids)	Cloning sites (5'/3')	Primers (forward) Primers (reverse)
GAL4-hEcd (1–644)	Sall/NotI	5'-AAGTCGACAAGATATCATGGAAGAAACCATGAAGCT-3' 5'-AAAGCGGCCGCTTAATTTTTTGTGGCTTACT-3'
GAL4-hEcd (1–610)	Sall/NotI	5'-AAGTCGACAAGATATCATGGAAGAAACCATGAAGCT-3' 5'-ACCGCGGCCGCTCAGGAGCTATAGGATTCCAA-3'
GAL4-hEcd (1–539)	Sall/NotI	5'-AAGTCGACAAGATATCATGGAAGAAACCATGAAGCT-3' 5'-AATGCGGCCGCAAGTGTCTTCAGGGAAGC-3'
GAL4-hEcd (1–155)	Sall/NotI	5'-AAGTCGACAAGATATCATGGAAGAAACCATGAAGCT-3' 5'-ACCGCGGCCGCTCATCCAGATTTTCTTGGTGC-3'
GAL4-hEcd (1–438)	Sall/NotI	5'-AAGTCGACAAGATATCATGGAAGAAACCATGAAGCT-3' 5'-ACCGCGGCCGCTCAAGACTCGGATTCTTAAAC-3'
GAL4-hEcd (150–438)	Sall/NotI	5'-AAGTCGACAAGATATCGCACCAAGAAAATCTGGA-3' 5'-ACCGCGGCCGCTCAAGACTCGGATTCTTAAAC-3'
GAL4-hEcd (150–644)	Sall/NotI	5'-AAGTCGACAAGATATCGCACCAAGAAAATCTGGA-3' 5'-AAAGCGGCCGCTTAATTTTTTGTGGCTTACT-3'
GAL4-hEcd (439–644)	Sall/NotI	5'-AAGTCGACAAGATATCGTTTCCAAGGAGGAGAAGGAGC-3' 5'-AAAGCGGCCGCTTAATTTTTTGTGGCTTACT-3'
Flag-hEcd (1–497)	EcoRV/NotI	5'-AAGTCGACAAGATATCATGGAAGAAACCATGAAGCT-3' 5'-ACCGCGGCCGCTTACCCTAAAATCTTATCAAAATA-3'
Flag-hEcd (1–480)	EcoRV/NotI	5'-AAGTCGACAAGATATCATGGAAGAAACCATGAAGCT-3' 5'-ACCGCGGCCGCTCATGGAGCCTCAGAAGGTTCTCG-3'
EGFP-hEcd (1–644)	SacI/SacII	5'-GAGGAGCTCACATGGAAGAAACCATGAAGCTTGC-3' 5'-AGACCGCGGATTTTTTGTGGCTTACTTGTGGTCTG-3'
EGFP-hEcd (1–438)	BspEI/S all	5'-GCTCCGGAGAAGAAACCATGAAGCTTG-3' 5'-CGGTCGACTTAAGACTCGGATTCTTTTTGCC-3'
EGFP-hEcd (150–644)	BspEI/S all	5'-GCTCCGGAGCACCAAGAAAATCTGGAGC-3' 5'-CGGTCGACTTAATTTTTTGTGGCTTACTTGTGG-3'

**Table 2** Primer sequences used in site-directed mutagenesis.

Mutation site (human Ecd)	Primers
I481A	5'-CCTTCTGAGGCTCCAGCTACTTTTGATGCAGAT-3' 5'-ATCTGCATCAAAAGTAGCTGGAGCCTCAGAAGG-3'
D484A	5'-GCTCCAATCACTTTTGCTGCAGATTCTTTCT-3' 5'-AGAAAAGAACTGCAGCAAAAAGTGATTGGAGC-3'
D484F	5'-CCAATCACTTTTTTGCAGATTCTTTT-3' 5'-AAAAGAATCTGCAAAAAAGTGATTGG-3'
S487A	5'-CACTTTTGATGCAGATGCTTTTCTTAATTATTTTG-3' 5'-CAAAATAATTAAGAAAAGCATCTGCATCAAAAAGTG-3'
L489A	5'-GATGCAGATTCTTTTGCTAATTATTTTGATAAG-3' 5'-CTTATCAAAATAATTAGCAAAAAGAACTGCATC-3'
D510R	5'-TCTGATGATCTGGATCGGGAAGACTTTGAATGT-3' 5'-ACATTCAAAGTCTTCCGATCCAGATCATCAGA-3'
D512R	5'-GATCTGGATGATGAACGCTTTGAATGTTTAGAT-3' 5'-ATCTAAACATTCAAAGCGTTCATCATCCAGATC-3'
D520P	5'-GTTTAGATAGTGATCCGGACTTGGACTTTG-3' 5'-CAAAGTCCAAGTCCGATCACTATCTAAAC-3'
ΔAR (502–532)	5'-ATTAGGCCTTGGCCCTAAAAT-3' 5'-GCTTCCCTGAAAGGAACACTT-3'

ment that might be caused by the insufficient ecdysone hormone production (Garen et al., 1977). However, several lines of evidence suggest other functions of Ecd in *Drosophila* and these defects cannot be fully explained by the low level of ecdysone in these mutant flies. To date, the precise biochemical function of Ecd in ecdysone pathway, such as an

enzyme function or other regulatory role, has not been elucidated. It is also important to note that the execution of ecdysone pathway in *Drosophila* constitutes transcription of various genes during embryonic development and together with the information presented here, it is reasonable to suggest that Ecd might play a role in transcription in flies.

Transcriptional regulator function requires localization in the nucleus; however, there is a discrepancy of Ecd subcellular localization observed in *S. pombe* vs. *Drosophila*. The majority of Ecd localizes to the cytoplasm in *Drosophila* (Gaziova et al., 2004); however, *S. pombe* Ecd localizes to the nucleus (Kainou et al., 2006). We analyzed the localization of hEcd in mammalian cells in detail. Although the steady-state distribution of Ecd is mainly cytoplasmic, we demonstrate that mammalian Ecd shuttles between the nucleus and cytoplasm and inhibition of its nuclear export by a CRM1 inhibitor led to the accumulation of Ecd in the nucleus. Thus, our data suggests that the localization of Ecd might be regulated under certain physiological conditions, which remains to be studied.

Through mutational studies, the region of Ecd responsible for nuclear export was determined. Dramatic change in subcellular localization of Ecd between truncated mutants aa 1–497 and aa 1–480 suggests that the region aa 480–497 harbors a potential NES sequence. Although the NES of Ecd does not precisely match the canonical NES sequences ( $\Phi$ -X<sub>(2–3)</sub>- $\Phi$ -X<sub>(2–3)</sub>- $\Phi$ -X- $\Phi$ ), the sequence ILNYFDKIL in the aa 488–496 region of Ecd represents a potential NES sequence.

Our extensive efforts to direct Ecd to the nucleus by fusing NLSs of SV40 large T antigen or GAL4-DBD with Ecd did not significantly alter Ecd localization, suggesting that Ecd is actively exported out of the nucleus. Furthermore, cytoplasmic localization of GAL4-hEcd suggests that Ecd localizes in the nucleus transiently, but this transient nuclear localization is apparently sufficient for Ecd to execute its role in transcriptional regulation as shown by the GAL4-reporter assay.

In conclusion, we have presented the biochemical characteristics of Ecd protein that support its function as a transcriptional regulator. At present, it is not known which transcription factor(s) associates with Ecd to exert its biological functions. Future studies will focus on the identification of primary transcription factors that directly associate with Ecd on the promoter. Such studies together with identification of Ecd target genes will shed light on the biological function of Ecd as a transcriptional regulator.

## Materials and methods

### Cell culture

293T cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen, ●●city? country?●●) supplemented with 10% Fetal Calf Serum (HyClone, ●●city? country?●●), 0.1 mM non-essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen) and 10 mM HEPES (Invitrogen). U2OS cells were grown in Alpha-Minimum Essential Medium (Invitrogen) supplemented with 10% Fetal Calf Serum, 0.1 mM MEM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES and 1 µg/ml insulin.

### Plasmids

For generating N-terminally tagged GAL4-DBD full-length or truncated forms (with amino acid numbers shown in parentheses) of hEcd, different primer sets (Table 1) were used to amplify by PCR the region of interest using Flag-hEcd plasmid (Zhang et al., 2006) as the template. Full-length mouse Ecd was amplified by RT-PCR using total RNA extracted from mouse embryonic fibroblast cells. Amplified PCR fragments were subcloned into SalI and NotI sites of pBIND vector (Promega, ●●city? country?●●). GAL4-hEcd (467–644) was generated from self-ligated GAL4-hEcd (439–644) construct followed by digestions with SalI and XhoI. For expression of N-terminal Flag-tagged Ecd constructs, different Ecd fragments were recovered from the corresponding GAL4-Ecd constructs with EcoRV and NotI digestions and subcloned into EcoRV and NotI sites of modified pcDNA3.1(+)-Flag1(Invitrogen) in which Flag sequence was added between NheI and HindIII sites by inserting annealed double-stranded oligonucleotides (5'-CTA GCA TGG ACT ACA AGG ACG ACG ATG ACA AGA-3' and 5'-AGC TTC TTG TCA TCG TCG TCC TTG TAG TCC ATG-3'). Flag-hEcd (1–497) and Flag-hEcd (1–480) were generated by PCR cloning. PCR primers and cloning sites are shown below. For EGFP-tagged hEcd constructs, PCR fragments of hEcd were cloned into pEGFP-C1 (Clontech, ●●city? country?●●). EGFP-hEcd (1–155) plasmid was constructed by subcloning the fragment (KpnI/Xba) of pcDNA3.1(+)-Flag-hEcd (1–155) into the KpnI and XbaI sites of pEGFP-C1 vector. EGFP-hEcd (150–438) and EGFP-hEcd (439–644) were also constructed by subcloning the fragment (EcoRI/Xba) of corresponding pcDNA3.1(+)-Flag-hEcd plasmids into the EcoRI and XbaI sites of pEGFP-C1 vector. NLSs of SV40 large T antigen were tagged by inserting annealed double-stranded oligonucleotides (5'-TCG ACA AGA TAT CCC AAA AAA GAA GAG AAA GGTA-3' and 5'-TAC CTT TCT CTT CTT TTT TGG GAT ATC TTG-3') into GAL4-hEcd (1–644) constructs digested with SalI and EcoRV. The fragment of NLS-hEcd (1–644) then recovered from GAL4-NLS-hEcd (1–644) by digesting with EcoRV and NotI was subcloned into the EcoRV and NotI sites of modified pcDNA3.1(+)-Flag1, thereby generating Flag-NLS-hEcd (1–644).

### Site-directed mutagenesis

The PCR-based site-directed mutagenesis method was used to generate various Ecd mutants. Approximately 5–50 ng of plasmid DNA was used as templates for PCR reaction (12–18 cycles, 1 min/kb of plasmid length for elongation times at 68°C) using *Pfu* turbo DNA polymerase (Stratagene, ●●city? country?●●) and specific primers. PCR products were treated with DpnI for 30 min to remove parental DNA and then used for transformation into *Escherichia coli*. The mutations were verified by sequencing. The primers for mutagenesis are shown in Table 2.

### GAL4-reporter gene assay

For reporter gene assay, the cells were plated in 24-well plates for 24 h and were then transfected with indicated pBIND fusion plasmid, and pG5/luciferase plasmid (Promega) using Lipofectamine 2000 (Invitrogen) or Eugene 6 (Roche, ●●city? country?●●) transfection reagent according to the manufacturer's instructions. GAL4-fused hEcd (200 ng) constructs were transfected in U2OS cells in 24-well plates together with 200 ng of GAL4-reporter plasmid (pG5/luciferase). Twenty hours after transfection, cells were lysed in 100 µl of passive lysis buffer (Promega). Luciferase activity, in



2–10  $\mu$ l of lysates, was measured using a luminometer and Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

### Immunofluorescence staining

Immunofluorescence staining of Flag-hEcd or GAL4-hEcd was performed using anti-Flag M2 mouse monoclonal antibody (Sigma, ●●city? country?●●) or anti-GAL4 mouse monoclonal antibody (Santa Cruz Biotechnology, ●●city? country?●●). Transfected cells on coverslips were fixed for 20 min in 1% paraformaldehyde, and then subjected to permeabilization [in phosphate buffered saline (PBS) containing 0.5% Triton X-100, for 15 min at room temperature (RT)] and blocking (in PBS containing 1% bovine serum albumin, for 30 min at RT). After blocking, cells were incubated for 1 h with anti-Flag antibody (1:1000) and then with anti-mouse IgG Alexa Fluor 488 or 564 (1:1000) (Molecular Probes, ●●city? country?●●) in PBS containing 0.1% Tween-20. After washing in PBS containing 0.1% Tween-20, cells on coverslips were mounted and visualized under fluorescence microscope. To block nuclear export of Ecd proteins, cells were treated with 5 ng/ml of LMB for 4 h.

### Western blotting and immunoprecipitation

Western blotting was performed using primary antibodies against Ecd (generated at the Monoclonal Antibody Facility at the Lurie Cancer Center, Northwestern University, Chicago, IL, USA), GAL4 (Santa Cruz Biotechnology), Flag (Sigma), HA (Sigma), PARP (Zymed, ●●city? country?●●), p300 (Santa Cruz Biotechnology),  $\alpha$ -tubulin (Sigma) and GFP (Santa Cruz Biotechnology). For immunoprecipitations, cell extracts were prepared in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40 and a protease inhibitor cocktail from Roche) and immunoprecipitated using 2  $\mu$ g of antibodies overnight at 4°C and the immunocomplexes were pulled down with protein A/G agarose (Santa Cruz Biotechnology) for an additional 2 h. Sample loading buffer (2 $\times$ ) was added to the purified immunocomplexes, boiled for 5 min and then loaded on SDS-PAGE. For biochemical fractionation of nuclear/cytoplasmic extracts, cells were lysed in hypotonic buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5% NP-40, 0.5 mM dithiothreitol, protease inhibitors). Nuclei were isolated by centrifugation of lysates at 1000 g for 5 min and supernatant was saved as cytosolic fraction. The cytosolic fraction was cleared by centrifugation at ●●●●g for 20 min. For nuclear fraction, nuclei were washed once with hypotonic buffer without NP-40 and lysed with high salt buffer containing 400 mM NaCl for 30 min on ice. Nuclear lysates were cleared by centrifugation at ●●●●g.

### Acknowledgments

We thank Dr. Xiangshan Zhao and other members of Band laboratory for helpful discussions and suggestions. This work was supported by the National Institutes of Health (NIH) Grant R01 CA96844-06, R01 CA94143 and Department of Defense grant W81XWH-07-1-0351 to V.B.; NIH CA87986, CA105489, CA99900, CA99163 and CA116552 to H.B. Y.Z. is a Carol and Marvin Gollob Fellow. J.H.K. is supported by a Korea Science and Engineering Foundation Grant (M06-2004-000-10547-0) and the Department of Defense Pre-doctoral Traineeship grant # W81XWH-08-1-0366. V.B. acknowledges the support of the Duckworth family through the Duckworth Family Chair for Breast Can-

cer Research. H.B. acknowledges the support from the Jean Ruggles-Romoser Chair for Cancer Research.

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Received July 31, 2009; accepted September 28, 2009